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**Karyotypic Analysis of
C-Banded Chromosomes of
Diploid Alfalfa: *Medicago
sativa* ssp. *caerulea* and ssp.
falcata and Their Hybrid**

G. R. Baughan and M. A. Hossain

Chromosomes of two diploid ($2n = 2x = 16$) subspecies of *Medicago sativa* ssp. *caerulea* and ssp. *falcata* and their hybrid were studied by C-banding. This study was undertaken to improve the C-banding technique for alfalfa chromosomes, develop a C-banded karyotype of the ssp. *caerulea* and ssp. *falcata*, and determine if the same C-banding technique could be

used to identify parental chromosomes in hybrids. The chromosomes of ssp. *falcata* have only centromeric bands and thus individual chromosomes could not be identified. One accession of ssp. *falcata* displayed an interstitial band in the middle of the long arm on the satellite chromosome. However, chromosome-specific bands were observed in ssp. *caerulea* enabling the identification of each of the eight pairs of chromosomes and the development of a idiogram. All chromosomes had centromeric bands and a terminal band in the short arm except the satellite chromosome (chromosome 8). Interstitial bands were also observed in the short arms, with the exception of chromosome 7. Chromosomes 1, 2, 3, and 8 each had one prominent interstitial band in their long arm. The satellited chromosome is easy to identify because of the presence of the secondary constriction, two bands located on either side of the nucleolar organizer region, and a large terminal band on its long arm. The differences in banding patterns between these subspecies allowed the identification of parental chromosomes in hybrid cells.

Cytogenetic research on alfalfa [*M. sativa* ssp. *sativa* (L.) L. & L.] and its closely related species has lagged behind other crops mainly due to four factors: (1) alfalfa chromosomes are very small, ranging from 2–3 μm in length in root tip cells; (2) the chromosomes are morphologically very similar; (3) cultivated alfalfa has a relatively high number of chromosomes ($2n = 4x = 32$); and (4) alfalfa is an autotetraploid with four nearly identical genomes complicating genetic analysis. Due to the autotetraploid nature of alfalfa, several researchers have chosen to study diploids. The diploid subspecies *Medicago sativa* ssp. *caerulea* (Less. ex Ledeb.) Schmalh. is considered to be the progenitor of cultivated tetraploid alfalfa. Cultivated alfalfa evolved from the diploid by sexual polyploidization via unreduced ($2x$) gametes which have been shown to occur in the diploid subspecies (McCoy and Bingham 1988; Pfeiffer and Bingham 1983). Bingham (1968), Bingham and Saunders (1974), and McCoy and Bingham (1988) have demonstrated that it is possible to transfer germplasm across ploidy levels.

M. sativa ssp. *falcata* (L.) Arcangeli has both diploid and tetraploid forms. The diploid subspecies is a small yellow flowered plant with straight to sickle-shaped pods as opposed to ssp. *caerulea* which is a small violet to lavender flowered plant

with coiled pods. Both diploid subspecies have been found growing wild in nature in the same geographical location and naturally occurring hybrids between them have been observed (Lesins and Lesins 1979; Small and Bauchan 1984). The primary center of diversity for the genus *Medicago* is found in the Caucasus, northwestern Iran, and northeastern Turkey (Ivanov 1977). *M. sativa* ssp. *falcata* has been shown to be a valuable germplasm source for the improvement of alfalfa because it has been the genetic source for extreme winter hardiness, broad crowns, creeping root habit, and some foliar disease resistance (Barnes et al. 1977). Rumbaugh (1991) showed that a single diploid accession of ssp. *falcata* exists in the pedigree of 30 alfalfa cultivars. Genetic and cytogenetic analysis of these two subspecies and their hybrid have shown that they are the same biological species (McCoy and Bingham 1991).

Karyotypic analysis from pachytene chromosomes of ssp. *caerulea* and ssp. *falcata* Arcangeli ($2n = 2x = 16$) has been conducted (Gillies 1968, 1970). Karyotypic analysis of somatic chromosomes of diploid ssp. *caerulea* has been accomplished through the use of an image analysis system (Bauchan and Campbell 1994). Despite these studies the identification of individual alfalfa chromosomes remains difficult.

There is only one report of successfully banding alfalfa chromosomes. Masoud et al. (1991) reported on the C-banded karyotype of *M. sativa* cv. CADL (cultivated alfalfa at the diploid level). However, they observed mostly centromeric and telomeric bands and only a few interstitial bands. Five annual *Medicago* species [*M. lesinsii* E. Small, *M. murex* Willd. (Mariani and Falistocco 1990), *M. noëana* Boiss. (Falistocco and Falcinelli 1993), *M. rugosa* Desr., and *M. scutellata* (L.) Miller (Mariani and Falistocco 1991)] have been studied and only centromeric bands were observed, thus providing information of little value for karyotypic analysis.

The present study was conducted to improve the C-banding technique chromosomes of alfalfa, develop a standard C-banded karyotype of the ssp. *caerulea* and ssp. *falcata*, and determine whether C-banding could be used to identify parental chromosomes in hybrids.

Materials and Methods

Six accessions of diploid ($2n = 2x = 16$) ssp. *caerulea* plus cv. CADL and eight ac-

Table 1. *Medicago sativa* ssp. *caerulea* and ssp. *falcata* germplasm used and the country of origin

Subspecies	Accession number	Country of origin
<i>caerulea</i>	PI 206453 ^a	Turkey
	PI 212798	Iran
	PI 243225	Iran
	PI 299046	Former Soviet Union
	PI 440507	Kazakhstan
	PI 464720	Turkey
<i>falcata</i>	cv. CADL ^b	USA
	PI 115365	Former Soviet Union
	PI 262332	Former Soviet Union
	PI 263154	Former Soviet Union
	PI 307398	Former Soviet Union
	PI 405064	USA
	PI 464728	Turkey
	PI 467970	USA
UAG 1806 ^c	Canada	

^a The plant introductions (PI) were obtained from the U.S. Plant Introduction Station in Pullman, Washington

^b cv. CADL provided by Ted Bingham, University of Wisconsin, Madison, Wisconsin.

^c UAG 1806 provided by the Karl Lesins Collection, University of Alberta, Edmonton, Canada.

cessions of ssp. *falcata* were studied. Refer to Table 1 for a list of the accessions used and their country of origin. Accessions PI 115365 and PI 263154 contain a mixture of diploid and tetraploid plants; only the diploid plants in these accessions were used. Hybrid seed was obtained from the cross between ssp. *falcata* (UAG 1806) and ssp. *caerulea* (cv. CADL) using vacuum suction hand emasculat.

Seeds were scarified and germinated on filter paper in Petri dishes at room temperature. Root tips were obtained 3 days after germination was initiated, pretreated in an ice bath for 20 h, and fixed in Farmer's fixative (3:1 v/v, 95% ethanol:glacial acetic acid) for at least 30 min. A modified improved C-banding technique (Hossain 1985) was utilized to band the chromosomes. A single fixed root tip was placed in a drop of 45% acetic acid for 2–3 min on a microscope slide. Dissection of dividing root tip cells was accomplished using sharpened needles under a dissecting microscope. Cells were gently warmed and squashed under a cover slip. The cover slip was removed after freezing the slide with liquid nitrogen. The slides were dried on a hot plate at 55°C–60°C for 10–12 min and then treated with 6% barium hydroxide for 5.5 min and briefly rinsed in distilled water. After rinsing, the slides were incubated in $2\times$ SSC (0.3 M sodium chloride plus 0.03 M trisodium citrate) at 60°C for 20 min. Slides were briefly rinsed in distilled water and stained in 7.3% Giemsa stain (Sigma) phosphate buffer (1 M NaH_2PO_4) at pH 6.8 for 30 min. The stained slides were briefly rinsed in distilled water,



Figure 1. C-banded chromosomes of *M. sativa* ssp. *falcata*. The bar represents 1 μ m.

air dried using a hair dryer for 2 min, and cover slips affixed using Permount. Twenty cells containing well-spread C-banded chromosomes were observed from each accession and the hybrid. Photomicrographs were taken using a Zeiss Axiophot Microscope using Kodak Technical Pan 2415 film. Photographs were printed on Agfa multigrade paper using high-contrast filters. The chromosomes from each cell were cut out and homologous pairs were sorted out to develop an idiogram based on length, centromere position, and banding pattern.

Results

Medicago sativa ssp. *falcata* C-banded chromosomes have bands only at the centromeric region, with the exception of the satellited (SAT) chromosome (chromosome 8), which also has a large band at the nucleolar organizer region (NOR) (Figure 1). Occasionally an interstitial band was observed in the middle of the long arm of the satellited chromosome. Without the diagnostic terminal or interstitial C-bands it is very difficult to karyotype the chromosomes of ssp. *falcata*. However,

M. sativa ssp. *caerulea* has several more bands than ssp. *falcata* in the accessions that were observed. In addition to the centromeric bands, which were the broadest of the C-bands, all of the chromosomes have telomeric bands in their short arms, all of the chromosomes except chromosome 7 have interstitial bands in their short arms, and chromosomes 1, 2, and 3 each have one prominent interstitial band in their long arms (Figure 2).

An idiogram of a standard karyotype is presented in Figure 3. A brief description of the C-banding pattern is as follows:

Chromosome 1: The largest chromosome without an NOR is submetacentric and has a terminal band and an interstitial band on the short arm; in addition to the centromeric band, a large interstitial band is located near the centromere on the long arm.

Chromosome 2: A submetacentric chromosome with a large telomeric band on the short arm and two interstitial bands located on each arm of the chromosome.

Chromosome 3: A submetacentric chromosome with an interstitial band close to the terminal band on the short arm; the interstitial band on the long arm is not as prominent as the one found on chromosome 1.

Chromosome 4: A submetacentric chro-

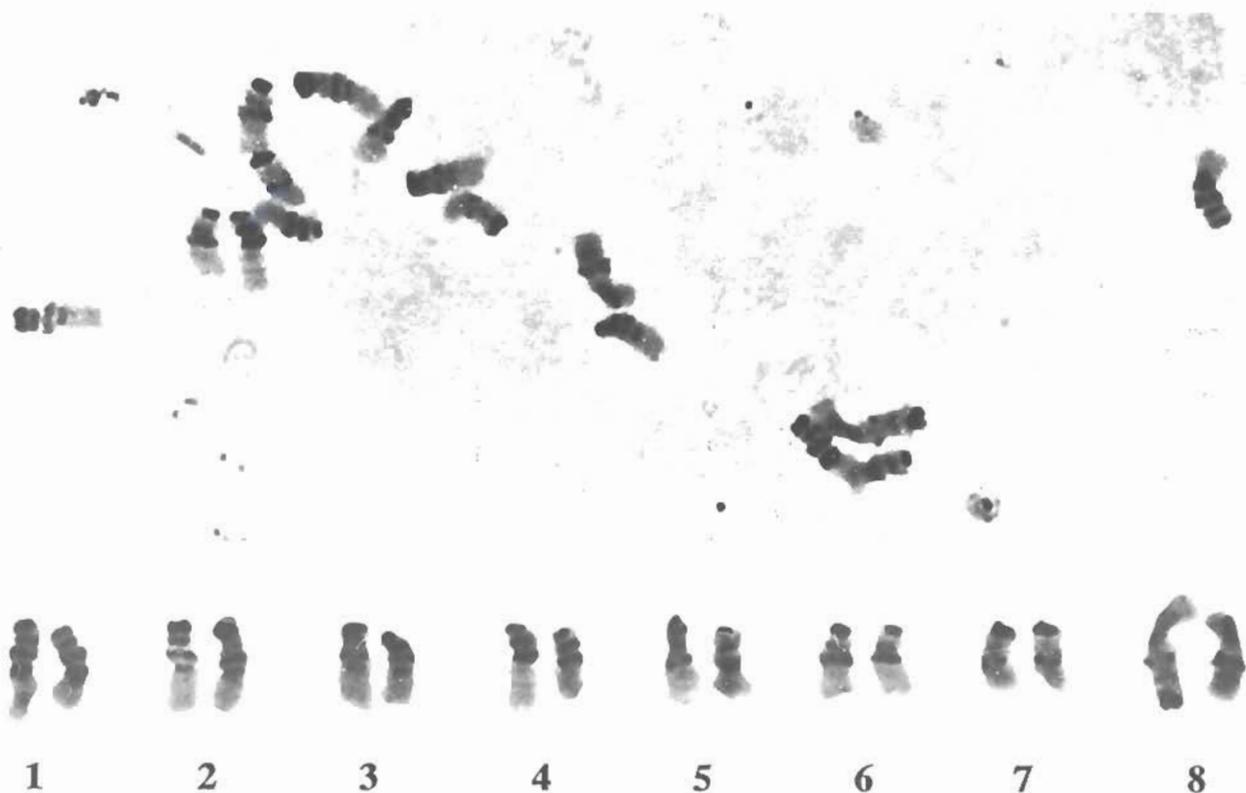


Figure 2. C-banded chromosomes of *M. sativa* ssp. *caerulea* and karyotype.

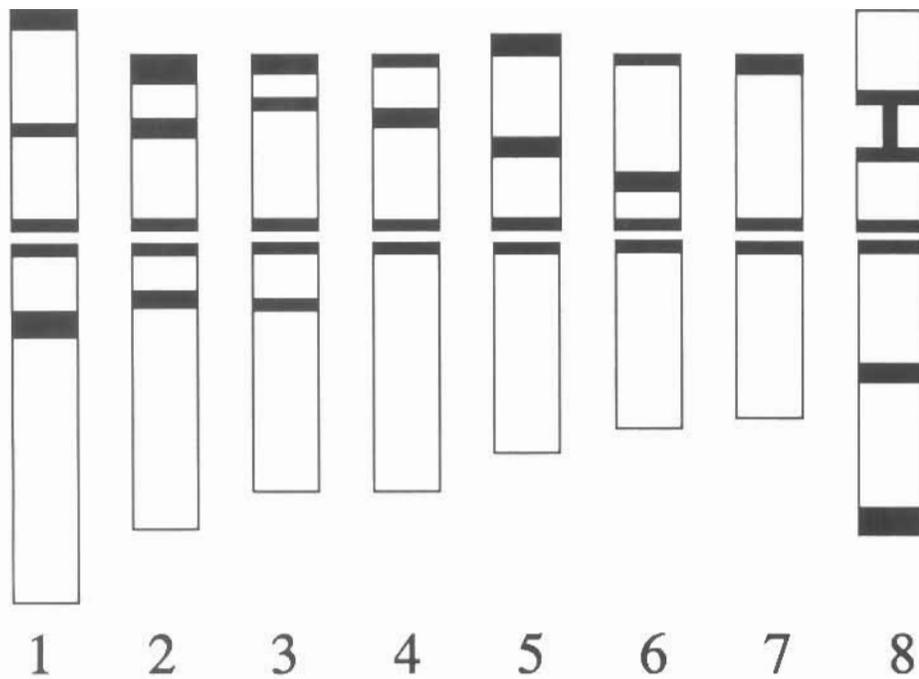


Figure 3. Idiogram of C-banded chromosomes of *M. sativa* ssp. *caerulea*.

Chromosome with an interstitial band midway between the telomeric band and the centromeric band. There were no interstitial bands located on the long arm, but occasionally a tertiary constriction can be found on the long arm of the chromosome.

Chromosome 5: A metacentric chromosome with an interstitial band closer to the centromeric band than the telomeric band on the short arm of the chromosome.

Chromosome 6: A short metacentric chromosome with a small terminal band and a prominent interstitial band on the short arm.

Chromosome 7: Another short metacentric chromosome with only centromeric bands and a telomeric band on the short arm of the chromosome, with no interstitial bands.

Chromosome 8: The SAT chromosome that is submetacentric with two bands flanked by the NOR and the centromere. A large terminal band is located on the long arm of the chromosome as well as an interstitial band.

Due the distinctive differences in the banding pattern of the two subspecies, *ssp. caerulea* having multiple bands and *ssp. falcata* having only centromeric bands, it was possible to identify the chromosomes of *ssp. caerulea* in the hybrid between the two subspecies (Figure 4).

Discussion

The karyotype of *ssp. caerulea* presented here varies from the C-banded karyotype described by Masoud et al. (1991). We found that all the chromosomes had centromeric bands and a terminal band in the short arm of all the chromosomes with the exception of chromosome 8, whereas their karyotype of cv. CADL (Masoud et al. 1991) has two chromosomes without telomeric bands. We also observed one interstitial band in the short arm of each chromosome except chromosomes 7 and 8, and chromosomes 1, 2, 3, and 8 each have a prominent interstitial band on their long arm that was not observed by Masoud et al. (1991).

The NOR is attached to the short arm of chromosome 8, which is in agreement with other karyotypes (Bauchan and Campbell 1994; Schlarbaum et al. 1988;). However, the karyotype developed by Masoud et al. (1991) shows the NOR on the long arm. We found that the pretreatment time of the root tips in the ice bath was critical for obtaining less contracted chromosomes for banding alfalfa chromo-



Figure 4. C-banded cell of a hybrid between *M. sativa* ssp. *falcata* and *M. sativa* ssp. *caerulea*. Arrows indicate the *M. sativa* ssp. *caerulea* chromosomes. The bar represents 1 μ m.

somes with interstitial bands. In chromosomes with excessive contraction the bands fuse together and only a few bands are actually detected. The SAT chromosome is easy to identify because of the presence of the secondary constriction and also because of the large terminal band on the long arm of the chromosome.

The distinctive C-banding pattern of *ssp. caerulea* chromosomes enabled us to develop a standard karyotype that may be helpful in studying cytogenetic and evolutionary relationships among species of *Medicago*. The differences we observed in the banding patterns of these two subspecies makes it possible to identify parental chromosomes in the hybrid.

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Corresponding Editor: Prem P. Jauhar