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

G. R. Bauchan 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. faucalata have only centromeric bands and thus individual chromosomes could not be identified. One accession of ssp. faucalata 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 karyogram. 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. Chromosome 1, 2, 3, and 8 each had one prominent interstitial band in their long arm. The satellite chromosome was 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 semilunar band on its long arm. The differences in banding patterns between these subspecies allow the identification of parental chromosomes in hybrid cells.

Cytogenetic research on allafia (M. afrobus ssp. satotum (L.) L. & L.) and its closely related species lagged behind other crops mainly due to four factors: (1) allafia chromosomes are very small, ranging from 2–3 μm in length in root tip cells; (2) the chromosomes are morphologically very similar; (3) cultivated allafia has a relatively high number of chromosomes (2n = 4x = 32); and (4) faucalata is an autotetraploid with four nearly identical genomes confounding genetic analysis. Due to the autotetraploid nature of allafia, several researches have chosen to study diploids. The diploid subspecies Medicago sativa ssp. caerulea (Less. ex. Ledeb.) Schmalz, is considered to be the progenitor of cultivated tetraploid allafia. Cultivated allafia evolved from the diploid by sexual polyploidization via induced (2n) gametes which have been shown to occur in the diploid subspecies (McCoy and Blangham 1988; Pfeiffer and Blangham 1985; Blangham 1988, Blangham and Saunders (1984), and McCoy and Blangham (1988) have demonstrated that it is possible to transfer germplasm across ploidy levels. M. sativa ssp. faucalata (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 (Leslie and Leslie 1979; Small and Bauchan 1984). The primary center of diversity for the genus Medicago is found in the Caspian, northern-western Iran, and northeastern Turkey (van Royen 1977). M. sativa ssp. faucalata has been shown to be a valuable germplasm source for the improvement of allafia because it has been the genetic source for: extreme winter hardiness, broad crowns, creeping root habit, and some foliar disease resistance (Barrenetxea et al. 1977). Run-baugh (1981) showed that a single diploid accession of ssp. faucalata exists in the pedigree of 30 allafia cultivars. Genetic and cytogenetic analysis of these two subspecies and their hybrid clones have shown that they are the same biological species (McCoy and Blangham 1991).

Karyotypic analysis from acheneotype chromosomes of ssp. caerulea and ssp. faucalata Arcangeli (2n = 2x = 16) has been conducted (Gillies 1995, 1997). 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 allafia chromosomes remains difficult.

There is only one report of successfully banding allafia chromosomes. Masoud et al. (1991) reported on the Chomsky karyotype of M. sativa cv. CADL (cultivated allafia at the diploid level). However, they observed mostly centromeric and telomeric bands and only a few interstitial bands. Five annual Medicago species (M. lentul E. Sm. M. arenul Willd., M. truncul and Falaiococco 1990), M. arenul Biuss, (Falaiococco and Falaioclico 1993), M. rugosul Desr., and M. scortetico (L.) Merian (Sarizai and Falaioclico 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 banding technique chromosomes of allafia, develop a standard C-banding karyotype of the ssp. caerulea and ssp. faucalata, 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 accessions of ssp. faucalata were studied. Refer to Table 1 for a list of the accessions used and their country of origin. Accessions PI 113365 and PI 206134 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. faucalata (UAG 1980) and ssp. caerulea (cv. CADL) using vacuum suction-based emasculation.

Seeds were harvested and germinated on filter papers in Petri dishes at room temperature. Root tips were fixed 3 days after germination was initiated, pretreated in an ice bath for 2–3 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 0.1 M acetic acid for 2–3 min on a microscope slide. Dissection of dividing root tip cells was accomplished using fine-tipped 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 55–60°C for 10–12 min and then treated with 0.1 barium hydroxide for 5.5 min and briefly rinsed in distilled water. After rinsing, the slides were incubated in 2 + 3C (0.3 M sodium chloride plus 0.03 M potassium 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 NaH2PO4) at pH 6.8 for 30 min. The stained slides were briefly rinsed in distilled water.

Table 1. Megaceropy ssp. caerulea and ssp. faucalata germplasm used and the country of origin

<table>
<thead>
<tr>
<th>Country of origin</th>
<th>Accession number</th>
<th>Subspecies</th>
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<tbody>
<tr>
<td>Iran</td>
<td>PI 113365</td>
<td>faucalata</td>
</tr>
<tr>
<td>Pakistan</td>
<td>PI 113365</td>
<td>faucalata</td>
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<tr>
<td>Turkey</td>
<td>PI 206134</td>
<td>faucalata</td>
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<tr>
<td>Turkmenistan</td>
<td>PI 206134</td>
<td>faucalata</td>
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<tr>
<td>China</td>
<td>PI 113365</td>
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<td>Turkey</td>
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<td>Iran</td>
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<tr>
<td>Pakistan</td>
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<td>United States</td>
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<td>China</td>
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The 70% ethanol-glacial acid (3:1) was obtained from the U.S. Place (Insects Stained at Franklin, Washington v/c CADL, provided by Ted Brigham, University of Wisconsin, Madison, Wisconsin). (1986) provided by the Earl Larson Collection (typo variety of Alberta, Idaho, Canada).
Results

Medicago sativa ssp. falcata C-banded chromosomes have bands only at the centromeric region, with the exception of the submetacentric (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. corvina 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-
moune 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. corallus* having multiple bands and *ssp. halocru* having only centromeric bands, it was possible to identify the chromosomes of *ssp. corallus* in the hybrid between the two subspecies (Figure 4).

**Discussion**

The karyotype of *ssp. corallus* 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-

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**Figure 3.** Diagram of C-banded chromosomes of *M. varico ssp. corallus*.

**Figure 4.** C-banded cells of a hybrid between *M. varico ssp. halocru* and *M. varico ssp. corallus*. Arrows indicate the *M. varico ssp. corallus* chromosome. The bar represents 1 μm.
somes with interstitial bands. In chromo-
somes 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 Cauding pattern of sat, excisoria chromosomes enabled us to develop a standard karyotype that may be helpful in studying cytogenetic and evo-
lutionary relationships among species of Medicago. The differences we observed in the banding patterns of these two subspe-
cies makes it possible to identify parental chromosomes in the hybrid.

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