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Distribution and Characterization of Heterochromatic DNA in the Tetraploid African Population Alfalfa Genome

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ABSTRACT

A reference karyotype of tetraploid alfalfa (*Medicago sativa* subsp. *sativa*, $2n = 4x = 32$) African Population germplasm source (PI 536539) was constructed using the combined techniques of C-banding and image analysis. The image analysis system is a method of obtaining enhanced images of chromosomes in which morphological data can be obtained for the development of karyotypes. In addition, C-banding is a diagnostic tool for distinguishing the homologous chromosomes of alfalfa. Chromosome analysis of tetraploid alfalfa revealed that alfalfa has four similar series of chromosomes based on their chromosome morphology and C-banding. The karyotype of African Population alfalfa germplasm consists of one set of chromosomes with satellites (SATs, Chromosome 8), four sets of submetacentric chromosomes (Chromosomes 1–4), and three sets of metacentric chromosomes (Chromosomes 5–7). All of the chromosomes have centromeric bands and a terminal band on the short arm, with the exception of the SAT. Interstitial bands were observed on the short arm of each of the chromosomes, with the exception of Chromosome 7. Chromosomes 1, 2, and 3 have interstitial bands on their long arms. There exists considerable variability in the number, intensity, and location of the constitutive heterochromatic DNA; however, this variability is not sufficient to preclude recognition of the homologous chromosomes. The chromosome banding pattern of the African Population germplasm resembles the distribution of heterochromatic DNA C-bands of diploid *M. sativa* subsp. *caerulea* (Less. ex Ledeb.) Schmalh. The African Population karyotype of alfalfa developed in this study is suggested as the reference for the development of additional chromosome maps of diverse alfalfa populations.

ALFALFA, a tetraploid ($2n = 4x = 32$), is the most important perennial forage crop grown in North America. It is recognized as a widely adapted agronomic crop, an effective source of biological N_2 fixation, an energy-efficient crop to grow, one of the highest sources of protein yield per hectare, and an attractive source of nectar for honey bees (*Apis mellifera* L., Barnes et al., 1988). Alfalfa is the third most widely grown crop in the USA after corn (*Zea mays* L.) and soybeans [*Glycine max* (L.) Merr.]. Alfalfa is a major component of pastures and is harvested as hay for animal feed.

There are nine historically distinct sources of alfalfa germplasm introduced into different regions of the USA between 1850 and 1947: African, Chilean, Flemish, Indian, Ladak, Peruvian, Turkistan, *M. falcata* L. [= *Medicago sativa* subsp. *falcata* (L.) Arcang.], and M. *varia* Martyn [= *Medicago sativa* nothosubsp. *varia* (Martyn) Arcang.] (Barnes et al., 1977). One of these sources was the African germplasm, introduced into the USA reportedly from Egypt in 1924 as 'Hegazi' (PI 31370, Barnes et al., 1977). African germplasm is a nondormant type of alfalfa which is present in ≈50% of the alfalfa grown in California (Barnes et al., 1977). In 1990, broad-based populations of the nine germplasm sources were registered with the African Population germplasm source identified as Reg. No. GP-238, PI536539 (Melton et al., 1990). The ancestry of this African Population germplasm source includes: 'Lew', 'Sonora', 'Sonora 70', Bryan Syn., 'African 4-31-A', 'Moapa', and 'African 4-44-A', which are cultivars or germplasm sources that were recognized as African. This germplasm source was developed by planting equal amounts of parental seed in a crossing block in Las Cruces, NM. Syn 1 seed was produced from this crossing block across two separate years in an isolation cage, by pollinating with honey bees. Additional seed was produced from the Syn 1 in Prosser, WA, to produce Syn 2 seed, which was made available for distribution. These germplasm sources were released for basic studies related to genetic diversity, heterozygosity, and heterosis in alfalfa. There have been only two studies conducted using these nine sources of germplasm: Kidwell et al. (1994) evaluated them for restriction fragment length polymorphism (RFLP) molecular marker differences, and Ray et al. (1998) evaluated them for forage yield, water-use efficiency, and canopy morphology.

Identification of alfalfa chromosomes at pachytene has been conducted by Gillies (1970) and Ho and Kasha (1974). Analysis of somatic chromosomes of tetraploid alfalfa previously has been reported (Agarwal and Gupta, 1983; Falistocco, 1987; Sclarbaum et al., 1988; Falistocco et al., 1995). Agarwal and Gupta (1983) karyotyped several *Medicago* species with chromosome measurements made using an ocular micrometer. Measuring chromosomes from photomicrographs, Falistocco (1987) obtained much larger chromosome measurements than what others have reported for the genus *Medicago*; for example, a total length ranging between 9 and 12 μm. Schlarbaum et al. (1988) karyotyped tetraploid alfalfa from plants that had been regenerated from a single cell protoplast in tissue culture; however, it is known that plants regenerated from a tissue culture system potentially can have chromosomes which have

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Abbreviations: NOR, nucleolar organizer region; RFLP, restriction fragment length polymorphism; SAT, chromosome with a satellite.

been altered (Bingham et al., 1988). Image analysis to obtain chromosome morphological measurements has been used to analyze the somatic chromosomes of diploid alfalfa, *M. sativa* subsp. *coerulea* (Bauchan and Campbell, 1994), and tetraploid alfalfa, *M. sativa* subsp. *sativa*. (Bauchan and Hossain, 2001).

Masoud et al. (1991) were the first to report the successful C-banding of diploid alfalfa chromosomes. They developed their karyotype on the basis of the C-banding pattern of *M. sativa* cv. CADL (Cultivated Alfalfa at the Diploid Level); however, most of the heterochromatic bands appeared at the centromeres and the telomeric ends, and few interstitial bands were observed. Recent studies have shown that it is possible to identify individual chromosomes of diploid accessions of *M. sativa* subsp. *coerulea* and subsp. *falcata* by their unique banding patterns using C-banding (Bauchan and Hossain, 1997, 1999a). Falistocco et al. (1995) reported the first C-banded karyotype of tetraploid alfalfa based on the Italian cultivar 'Turrena'.

The objectives of this study were to determine the distribution of constitutive heterochromatic DNA, characterize the polymorphisms which might exist, and develop a reference karyotype of tetraploid alfalfa based on the nondormant germplasm source African Population, utilizing the combined techniques of C-banding and image analysis.

MATERIALS AND METHODS

Seeds of African Population germplasm were obtained from the U.S. Plant Introduction Station in Pullman, WA. Twenty-five plants were grown in the greenhouse to determine if indeed the seed source was true to phenotype for African Population alfalfa. Seeds were scarified and germinated in Petri dishes at 25 °C on filter paper. Root tips obtained from roots 5 to 10 mm in length 2 to 3 d after germination were pretreated in an ice bath for 18 h before fixation in Farmer's Fixative (3:1 v/v, 95% ethanol:glacial acetic acid). C-banding was conducted according to Bauchan and Hossain (1997). Ten cells per plant that possessed well-spread C-banded chromosomes were observed in 25 individual plants. Observations were made using a Zeiss Axiophot Microscope (Carl Zeiss, Inc., Thornwood, NY)[1] with a computerized image analysis system attached to the microscope. Photomicrographs were taken using Kodak Technical Pan Film (Eastman Kodak Company, Rochester, NY).

Karyotypic analysis was initiated through the use of a PC computer-based KARYOTYPER software module of the Loats Associates, Inc. (Westmenster, MD) INQUIRY image analysis system to obtain morphometric measurements of each chromosome (Bauchan and Hossain, 2001). Briefly, all the measurements were made interactively using a mouse while viewing the images on the screen. The centromere for each chromatid was identified and each arm of the chromatid was measured starting with the short arm and then the long arm. Each chromosome was measured systematically until all the chromosomes had been measured. The chromosome arm lengths were averaged together and the arm ratio (long arm:short arm average), total chromosome length (short arms + long arms), and relative chromosome lengths (length of the individual chromosome/total length of all chromosomes in the genome) were calculated. The chromosome homologs were determined by sorting the data based on relative chromosome length, and the chromosomes were arranged from longest to shortest, with the SAT placed following the shortest chromosome. Adjustments were made to the karyotype if the chromosome was distorted during the squashing process or according to its characteristic banding patterns.

The efficacy of the image analysis system to differentiate between alfalfa chromosomes was tested on 25 individuals. Statistical analysis of the data using Tukey's test was accomplished using SAS (1998). Idiograms were developed for each plant which displayed polymorphisms. Figures of the chromosomes were made using Adobe Photoshop.

RESULTS AND DISCUSSION

The 25 plants grown in the greenhouse were determined by observation of the growth habit, flower color, and pod type to fit the African Population phenotype. The karyotype of the tetraploid African Population germplasm source consisted of one set of homologous SATs (Chromosome 8), four sets of submetacentric chromosomes (Chromosomes 1–4) and three sets of metacentric chromosomes (Chromosomes 5–7). The image analysis system is a method of obtaining quality images of chromosomes through its enhancement capability. Enhancement of the chromosomes by pseudocoloration and enlargement of the images enable the edges of the chromosomes and the heterochromatic bands to be distinguished for easy identification and measurement (Fig. 1). The efficacy of the image analysis system for discriminating among chromosome sets based on Tukey's test (SAS, 1998), as determined by this study and previous studies (Bauchan and Campbell, 1994; Bauchan and Hossain, 2001), showed that the only measurement which could be used reliably to distinguish the chromosomes was the relative chromosome length. The coefficients of variation indicate that parameter estimation was reasonably precise (Table 1).

The C-banding pattern is diagnostic in distinguishing the chromosomes based upon the position and intensity of the bands, particularly in the identification of the submetacentric (Chromosomes 1–4) and the metacentric (Chromosomes 5–7) chromosomes. Comparison of the C-banding pattern to the pachytene karyotypes of Gillies (1970) and Ho and Kasha (1974) revealed that the telomeric and interstitial C-bands that were observed on the short arms of the chromosomes in this study were in locations similar to the knobs they observed at pachytene. All of the cells within an individual plant had the same banding pattern. The broadest of the C-bands in all the chromosomes were located at their centromere. Nine of the 25 plants studied had the same banding pattern as was observed in diploid *M. sativa* subsp. *coerulea* (Bauchan and Hossain, 1997). However, there were twice the number of chromosomes, and each homologous set contained four nearly identical chromosomes (Fig. 1). The karyotype of the African Population germplasm was chosen by our laboratory as the reference karyotype for alfalfa because in preliminary studies

[1] Mention of a trade name or proprietary product does not constitute a guarantee, warranty, or recommendation of the product by the USDA, and does not imply its approval to the exclusion of other products that may also be suitable.

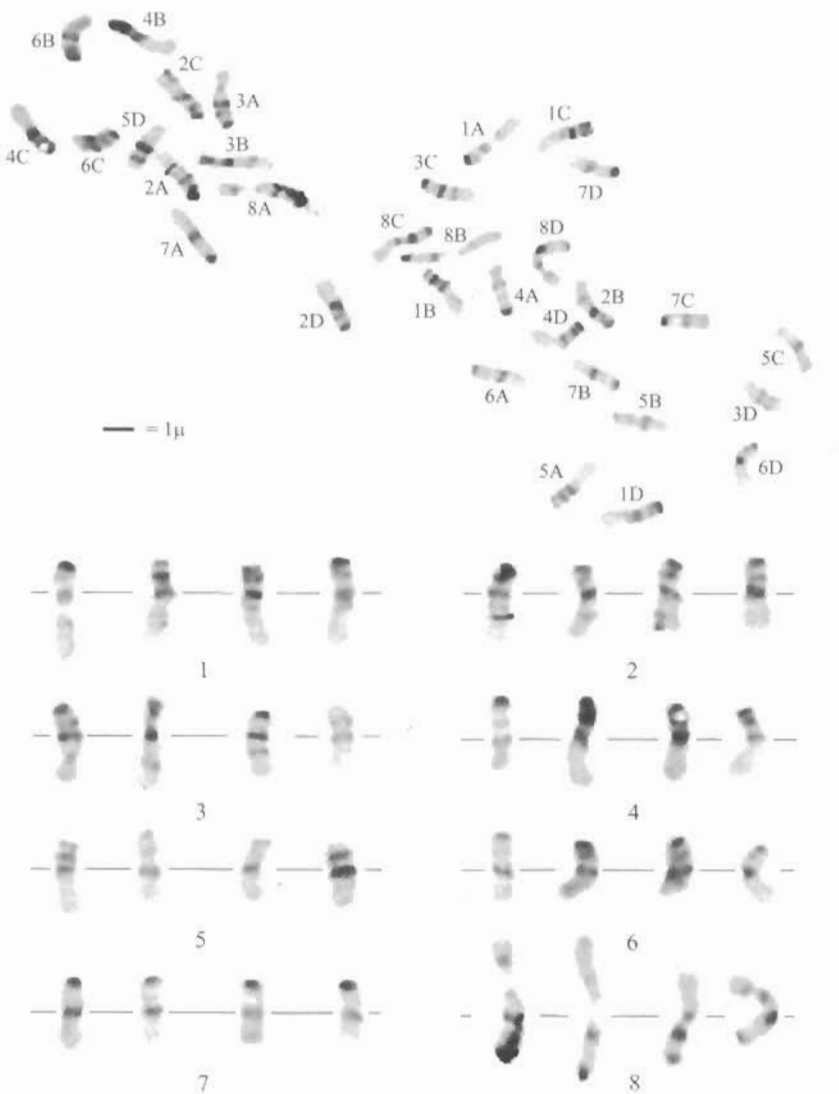

Fig. 1. C-banded karyotype of *Medicago sativa* subsp. *sativa*, African Population. The bar represents 1 μm.

(Bauchan and Hossain, 1998), of all of the nine germplasm sources, African Population contained the largest number of heterochromatic bands and represents the best fit to the C-banding pattern observed in diploid of *M. sativa* subsp. *coerulea* (Bauchan and Hossain, 1997)

Polymorphisms in C-banded constitutive heterochromatic DNA were found to exist among individuals in the African Population germplasm source. The reference karyotype is represented in Fig. 1.

Chromosome Descriptions

Chromosome 1. The largest chromosome with an average length of 2.46 μm (Table 1) without a nucleolar

Table 1. Efficacy of the image analysis system for differentiating among homologous chromosome sets in alfalfa for 25 cells.

Chromosome set	Average short arm	Average long arm	Arm ratio	Average total length	Relative length (%)†	Average SAT length
	μ					
1	1.01 ± 0.03a‡	1.45 ± 0.03a	1.44 ± 0.01a	2.46 ± 0.03b	13.70 ± 0.03b	
2	1.01 ± 0.02a	1.35 ± 0.03a,b	1.34 ± 0.01a	2.36 ± 0.02c	13.14 ± 0.03c	
3	0.99 ± 0.03a	1.29 ± 0.02b	1.30 ± 0.01a	2.28 ± 0.03d	12.69 ± 0.02d	
4	0.95 ± 0.03a,b	1.24 ± 0.02b,c	1.31 ± 0.01a,b	2.19 ± 0.03e	12.19 ± 0.02e	
5	0.94 ± 0.03a,b	1.20 ± 0.03c,d	1.28 ± 0.01b,c	2.14 ± 0.03e	11.92 ± 0.02f	
6	0.89 ± 0.02b,c	1.12 ± 0.03d,e	1.26 ± 0.01c	2.01 ± 0.03f	11.19 ± 0.03g	
7	0.87 ± 0.03b,c	1.09 ± 0.03d,e	1.25 ± 0.01c	1.96 ± 0.02f	10.91 ± 0.03h	
8	0.84 ± 0.02c	1.06 ± 0.03e	1.26 ± 0.01c	2.56 ± 0.02a	14.25 ± 0.02a	0.66 ± 0.03
CV	10.01	11.52	8.76	9.37	7.45	

† Relative length as a percentage of total length of all eight chromosomes.
‡ Means not followed by the same letter are significantly different at $P < 0.05$ based on Tukey's test.

organizer region (NOR) is submetacentric; it 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. Two individual plants had a double interstitial band on the long arm of one of the homologous chromosomes. The interstitial band on the long arms was missing in one plant.

Chromosome 2. A submetacentric chromosome with a large telomeric band on the short arm and two interstitial bands located on both arms of the chromosome. This chromosome contains a large number of polymorphisms. A double interstitial band was observed in one homolog in one plant. Sixteen percent of the chromosomes observed were missing the interstitial band on the short arm in at least one chromosome and sometimes on all four homologs.

Chromosome 3. A submetacentric chromosome with an interstitial band close to the terminal band on the short arm. The interstitial bands on the long arms were not as prominent as the band found on Chromosome 1, and are located closer to the terminal end of the long arm. This chromosome contained the largest amount of polymorphisms in the genome. The most frequent polymorphism (24%) was the loss of the interstitial bands on the long arms on all four homologs. Twenty percent of the plants observed were missing the interstitial bands on the long arms for either two or three of the homologs. The interstitial bands on the short arms also were missing in six of the 25 individuals observed; sometimes (8%) all four homologs were missing the interstitial band, and in other instances (12%) only one homolog was missing its interstitial band. Occasionally (3%), a terminal band was missing on the short arm.

Chromosome 4. A submetacentric chromosome with an interstitial band midway between the telomeric band and the centromeric band on the short arm. There were no interstitial bands located on the long arm; however, occasionally a tertiary constriction was evident on the long arm of the chromosome. This chromosome did not contain any polymorphisms among the individuals observed, and all of the homologs appeared to be very similar.

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 with very few polymorphisms. Only two individuals were missing either the terminal band or the interstitial band but only on one homolog.

Chromosome 6. Another metacentric chromosome with a small terminal band and a prominent interstitial band on the short arm. A missing interstitial band on the short arm was the most frequent (20%) polymorphism observed. One to four chromosomes were missing this interstitial band in 28% of the plants. In two of the 25 plants, the terminal band was missing from the short arm.

Chromosome 7. The shortest chromosome in the genome [an average length of 1.96 μm (Table 1)] was a metacentric chromosome with only centromeric bands and a telomeric band on the short arm of the chromosome, with 5% of the plants possessing interstitial band on the short arm. The most common polymorphism (10%) was the loss of the terminal band on the short arm; however, this usually occurred only on one of the homologs.

Chromosome 8. The only SAT chromosome, with bands flanking the NOR and the centromere; a large terminal band as well as an interstitial band were located on the long arms of the chromosome. Almost half (48%) of the plants were found without the interstitial band on the long arm. The interstitial band was sometimes (12%) missing from all three homologs; in other instances (16%) it was missing on two homologs; and yet with others (20%), one homolog was missing the interstitial band. In two of the 25 plants, a double interstitial band was found on the long arm. Four percent of the chromosomes were missing the terminal band on the long arm of the chromosome. Figure 2 represents the summarization of the polymorphisms which were observed for each chromosome. A composite idiogram for the African Population germplasm source was constructed (Fig. 3).

There was considerable variability in the number, intensity, and location of the constitutive heterochromatic DNA found in alfalfa. In the alfalfa genome, the constitutive heterochromatic DNA was located mainly around the centromeres and the short arms of the chromosomes. The origin of additional bands is uncertain. They may have preexisted, or they could have resulted from reduplication of highly repetitive DNA, unequal crossing over, or possibly a translocation, as has been reported in alfalfa (Stanford and Clement, 1958). The loss of a terminal band can occur through a deletion or possibly by outcrossing with *M. sativa* subsp. *falcata*. Bauchan and Hossain (1997 and 1999a) showed that diploid *M. sativa* subsp. *falcata* chromosomes possess bands primarily at the centromeres. Preliminary studies of tetraploid *M. sativa* subsp. *falcata* chromosomes revealed that there were a larger number of C-bands than had been discovered in diploid *M. sativa* subsp. *falcata*; however, there were fewer bands than found in *M. sativa* subsp. *sativa* (Bauchan and Hossain, 1998, 1999b). Hybridization between M. *sativa* subsp. *falcata* and *M. sativa* subsp. *sativa* can occur naturally, and thus meiotic crossing over can take place, resulting in the loss of constitutive heterochromatic DNA.

Molecular studies of the nine germplasm sources of alfalfa by Kidwell et al. (1994) used RFLP patterns to characterize the germplasm sources. They also found much variability, even within a given germplasm source. They were unable to distinguish most of the germplasm sources from each other through the use of molecular markers and multivariate analysis, with the exception that *M. sativa* subsp. *falcata* and *M. sativa* subsp. *sativa* Peruvian Population formed two unique clusters.

This present investigation of tetraploid African Population alfalfa supports the conclusion of other researchers based on genetic studies (reviewed by McCoy and Bingham, 1988; and Rumbaugh et al., 1988) that alfalfa is an autotetraploid. The similarity of the chromosome morphology and the C-banding pattern (Fig. 1) among

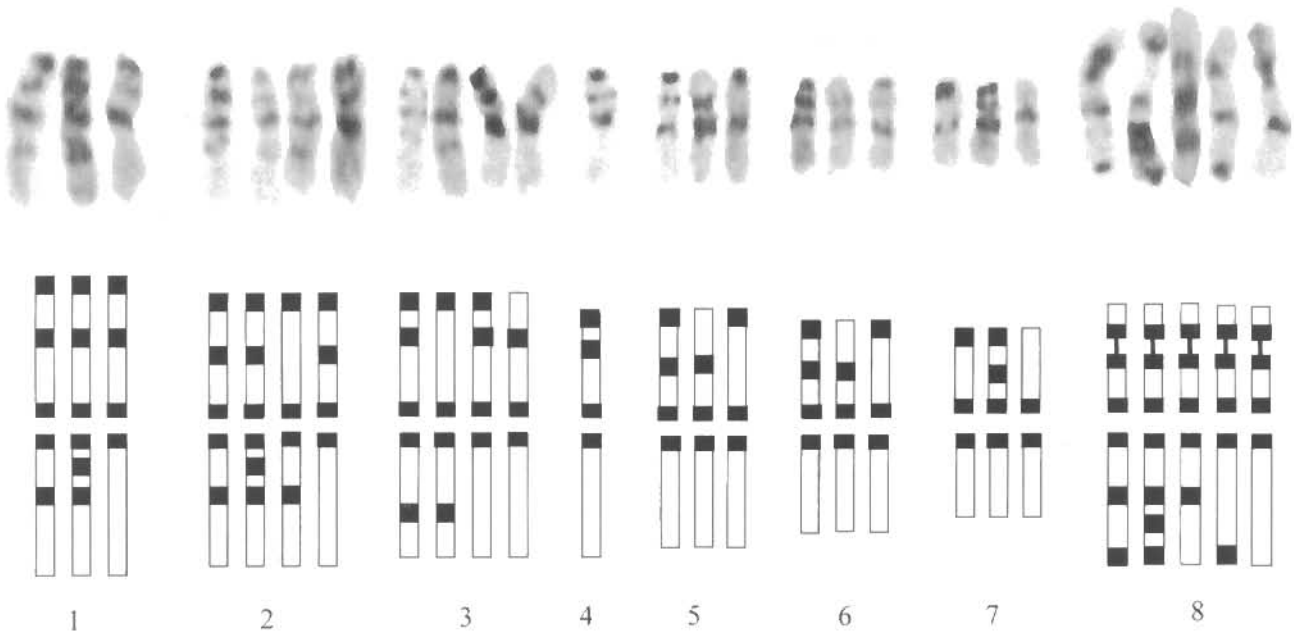

Fig. 2. Summary idiogram of the polymorphisms which were observed in the C-banded chromosomes of *Medicago sativa* subsp. *sativa*, African Population.

homologs made it possible to group alfalfa chromosomes into eight sets of four chromosomes. Additional evidence to show that tetraploid alfalfa is an autotetraploid is that the C-banding pattern of tetraploid alfalfa depicted in the idiogram (Fig. 3) is nearly identical to that of its putative progenitor diploid *M. sativa* subsp. *coerulea* (Bauchan and Hossain, 1997). The nondormant African Population germplasm source appeared to contain more subsp. *sativa* germplasm than subsp. *falcata*, as indicated by the consistency with which all four homologs possessed bands at the same location (Bauchan and Hossain, 1998, 1999b).

The tetraploid alfalfa karyotype presented in this study differs from the karyotype published by Falistocco et al. (1995), primarily in the greater number of bands than they observed. A number of reasons could explain the differences. First, they used a saturated solution of α-bromonapthalene for 4h as a pretreatment. Our experience has shown that an ice water pretreatment for 18h gives the largest number of bands, and thus the

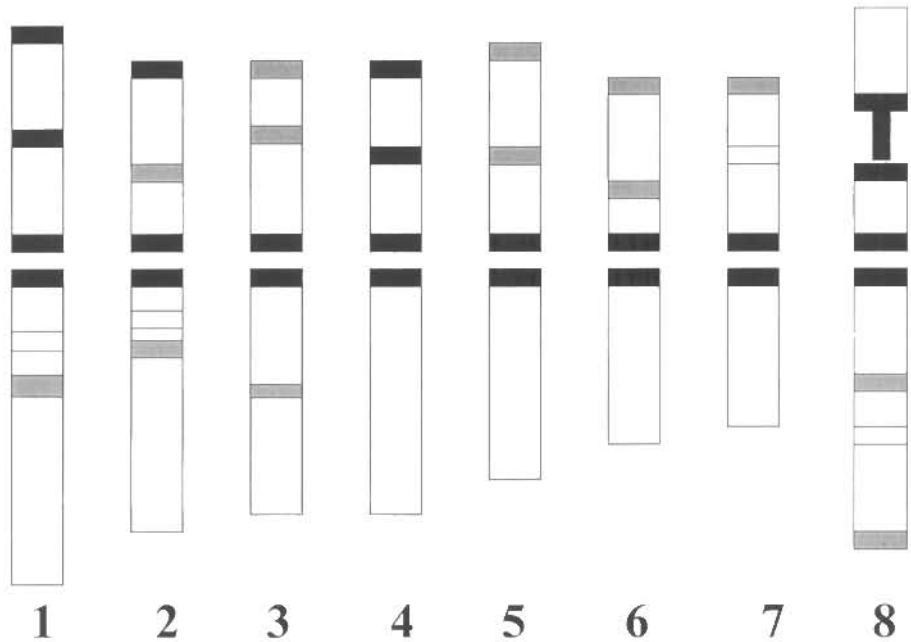

Fig. 3. A composite idiogram for the C-banded chromosomes of the *Medicago sativa* subsp. *sativa*, African Population germplasm source. Solid bands represent bands which were always present in all four homologs. Shaded bands represent bands which were not always present in all four homologs. Open bands represent bands which occasionally were present (<4%).

ability to distinguish the individual chromosomes from each other more critically, based on the larger number of landmark bands. Two bands which are located next to each other may appear as a single band if the chromosomes are too contracted, thus reducing the number of observable bands. Second, they used the Italian cultivar 'Turrena'. This cultivar may have a reduced amount of constitutive heterochromatic DNA compared with the African Population germplasm source used in this study. In preliminary cytogenetic studies of the nine germplasm sources (Bauchan and Hossain, 1998), we found that the more fall dormant germplasm sources contained a smaller amount of constitutive heterochromatic DNA. Therefore, if Turrena is a less dormant type alfalfa than the African Population, it may have a reduced number of C-bands.

This research demonstrated that, through the combined use of C-banding and image analysis techniques, it is possible to identify individual alfalfa chromosomes for the development of a karyotype of tetraploid alfalfa. The existence of considerable polymorphisms in the number, intensity, and location of the constitutive heterochromatic DNA may be due to the out-crossing nature of alfalfa. The C-banded chromosome map of alfalfa developed in this study is suggested as the reference for the development of additional chromosome maps of diverse alfalfa populations.

The utilization of C-banding and image analysis can have multiple applications in the field of cytogenetics and plant breeding, such as comparing evolutionary relationships and genome correspondence analysis among and between *Medicago* species, identification of chromosomal modifications, development of aneuploids for genetic studies, and the identification and incorporation of alien genetic material from wild species into alfalfa for alfalfa improvement.

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