

Chromosomal Polymorphism as Detected by C-Banding Patterns in Chilean Alfalfa Germplasm¹

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ABSTRACT

A cytogenetic investigation was conducted on the tetraploid alfalfa [*Medicago sativa* subsp. *sativa* (L.) L. & L.] Chilean germplasm source PI 536534 using the combined techniques of C-banding and image analysis. Cluster and multiple correspondence analyses were utilized to compare the C-banding patterns of the Chilean germplasm source and the previously published African germplasm source. Cytogenetic analyses revealed polymorphisms for heterochromatic DNA in the 19 plants observed in detail. Abundant variability in the number, intensity, and location of constitutive heterochromatic DNA was noted; however, this variability was not sufficient to preclude recognition of homologous chromosomes. Five out of the 50 plants studied were aneuploids ($2n = 4x + 1 = 33$ or $2n = 4x - 1 = 31$) because of the presence or absence of a chromosome with a satellite. The Chilean karyotype resembled the reference tetraploid African alfalfa karyotype; however, a reduction in the total amount of heterochromatic DNA was observed. Cluster analysis and multiple correspondence analysis based on all eight alfalfa genome chromosomes yielded no clear separation of Chilean and African germplasms. However, the analysis of C-banding patterns of Homolog 1 of Chromosome 8 in Chilean and African germplasms was effective in separating the two germplasm sources with the exception of two individuals from each germplasm source which clustered together.

ALFALFA IS THE MOST important forage legume crop grown in North America and the third most widely grown crop in the USA (Barnes et al., 1988). Alfalfa is primarily harvested as hay for animal consumption and is one of the major component of pastures. It is broadly adapted, energy efficient, and an excellent source of protein. It fixes nitrogen symbiotically, improves soil tilth, and is an attractive source of nectar for honey bees.

Cultivated alfalfa is an autotetraploid with 32 chromosomes ($2n = 4x = 32$). Most alfalfa cultivars trace to nine historically recognized germplasm sources: African, Chilean, Flemish, Indian, Ladak, Peruvian, Turkistan, *M. falcata*, and/or *M. varia* germplasm sources, which were introduced into different regions of the USA between 1850 and 1947 (Barnes et al., 1977). Chilean alfalfa was introduced into the new world by the Spaniards in the 16th century. Early introductions of alfalfa spread throughout South America and into the USA through Mexico. During the 1850s, Chilean sources were introduced into the southwestern USA and became the primary constituent in California, New Mexico, Oklahoma, and Kansas Common alfalfas (Barnes et al., 1977). In 1990, broad-based populations of the nine germplasm sources were registered with the Chilean

germplasm source identified as Reg. No. GP-233, PI 536534 (Melton et al., 1990), with an ancestry which includes cultivars Caliverde, California Common 49, California Common, Chilean Common, Chilean 21-5, and Chilean 21-5-5. 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 over two separate years in an isolated cage by means of honey bees (*Apis mellifera* L.). Additional seed was produced from the Syn 1 at Prosser, WA, to produce Syn 2 seed, which was released for basic studies related to genetic diversity, heterozygosity, and heterosis in alfalfa.

Several cytogenetic studies of somatic chromosomes of tetraploid alfalfa have been conducted (Agarwal and Gupta, 1983; Falistocco, 1987; Sclarbaum et al., 1988; Falistocco et al., 1995; Bauchan and Hossain, 2001a,b). Chromosome banding studies have shown that it is possible to identify individual chromosomes of *M. sativa* subspecies, including *coerulea*, *falcata* (Bauchan and Hossain, 1997, 1998a, 1999a), and *sativa* (Falistocco et al., 1995; Bauchan and Hossain, 2001a,b). Falistocco et al. (1995) reported the first C-banded karyotype of tetraploid alfalfa for the Italian cultivar Turrena. Bauchan and Hossain (2001b) evaluated the C-banding pattern of the African germplasm source of tetraploid alfalfa and concluded it resembled the C-banding pattern of diploid *M. sativa* subsp. *coerulea* (Bauchan and Hossain, 1997). The African germplasm source has revealed the maximum amount of heterochromatic DNA of the nine alfalfa germplasm sources studied (Bauchan and Hossain, 1998b). Also, the African C-band karyotype has been proposed as the reference karyotype for use in developing additional karyotypes of diverse alfalfa populations (Bauchan and Hossain, 2001b).

The objectives of this study were to characterize the distribution of constitutive heterochromatic DNA in tetraploid Chilean germplasm, identify chromosomal abnormalities, develop a karyotype of Chilean alfalfa, compare the Chilean karyotype with the reference African karyotype, and determine if cluster and multiple correspondence analyses could be used to distinguish chromosomes of different germplasm sources.

MATERIALS AND METHODS

Karyotype Analyses

Seeds of *M. sativa* subsp. *sativa* Chilean germplasm source PI 536534 were obtained from the U.S. Plant Introduction Station at Pullman, WA. Twenty-five plants were grown in

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the greenhouse to determine if the seed source was true to form for Chilean type alfalfa. Seeds were scarified and germinated in Petri dishes at 25°C on filter paper. Root tips were obtained from roots which were 5 to 10 mm in length 2 to 3 d after germination and 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) and 10 cells per plant were observed in 50 individual plants. Sufficiently spread C-banded chromosomes were obtained in 19 plants and were karyotyped. Observations were made with a Zeiss Axiophot Microscope (Carl Zeiss, Inc., Thornwood, NY) with an attached computerized image analysis system. Photomicrographs were taken with Kodak Technical Pan Film (Eastman Kodak Company, Rochester, NY) and karyotypic analyses were initiated by means of the Karyotyper software module of the INQUIRY image analysis system (Loats Associates, Inc., Westminster, MD) to obtain morphometric measurements of each chromosome (Bauchan and Hossain, 2001a,b).

The efficacy of the image analysis system to differentiate between alfalfa chromosomes was tested on the 19 individuals. Statistical analysis of the data by Tukey's test was accomplished by means of SAS (1999). Adjustments were made to the karyotype if the chromosome was distorted during the squashing process and/or according to banding patterns. Idiograms were developed for each plant which displayed polymorphisms. Figures of the chromosomes were made with Adobe Photoshop (Adobe Systems, Inc., San Jose, CA).

Statistical Analyses of C-Banding Patterns

Cluster and multiple correspondence analyses were used to determine whether or not C-banding patterns could be used to separate Chilean from African germplasm sources. Homolog 1 was judged to be the most definitive of the four homologs. By convention, Homolog 1 was the homolog with the largest number of bands of the homolog set. Each chromosome × homolog number 1 combination was scored separately for presence or absence of each C-band over the 19 Chilean and 17 African clones (Bauchan and Hossain, 2001b), and a combined presence/absence (1/0) matrix was computed.

Genetic distances (GDs), based on all pairwise comparisons for the eight chromosomes together and each chromosome separately, were computed by the formula $GD = 1 - [2N / (N_i + N_j)]$, where N is the number of common bands and N_i and N_j are the total number of bands for clones i and j (Nei and Li, 1979). This formula disregards 0/0 matches, which could be caused by factors other than genetic similarity, and weighs 1/1 matches by a factor of 2 to better separate genotypes.

A multiple correspondence analysis was performed on the GD data by means of PROC CORRESP (SAS Institute, 1999). This analysis produces weighted principal components (dimensions) from a contingency table assuming Euclidean distances. Clones were clustered by Ward's Minimum Variance (PROC CLUSTER, SAS Institute, 1999) where the distance between two clusters is the analysis of variance sum of squares between the clusters added over all variables. Genetic distances were squared prior to invoking the procedure. For interpretation, the sums of squares are converted to R^2 values. Ward's Minimum Variance method tends to join clusters with small numbers of observations and is biased toward producing clusters with roughly the same number of observations. Dendrograms were produced by PROC TREE and enhanced by PROC GPLOT (SAS Institute, 1999). Ward's procedure was also bootstrapped for Chromosome 8 data (100 cycles) by means of a program written in the Interactive Matrix Lan-

guage (SAS Institute, 1999) to produce percentages of identical clusters.

RESULTS AND DISCUSSION

Observation of the growth habit, flower color, and pod type of 25 Chilean plants grown in the greenhouse were judged to fit the Chilean alfalfa phenotype. Root tips of plants from this seed source were then used for characterization of the chromosomes of the Chilean germplasm. The image enhancement capability of the image analysis system enabled quality images of chromosomes to be obtained. 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 identification and measurement. The Chilean karyotype consisted of one set of homologous chromosomes with a satellite (Chromosome 8), four sets of submetacentric chromosomes (Chromosome 1–4) and three sets of metacentric chromosomes (Chromosomes 5–7) (Fig. 1). The Chilean karyotype was similar to the reference African karyotype (Fig. 2); however, as with earlier studies (Bauchan and Hossain, 1998b, 2001b), the total number of heterochromatic bands for African germplasm was larger, ranging from 92 to 108 compared with a range of 88 to 104 for Chilean germplasm. All of the cells within an individual Chilean plant had the same banding

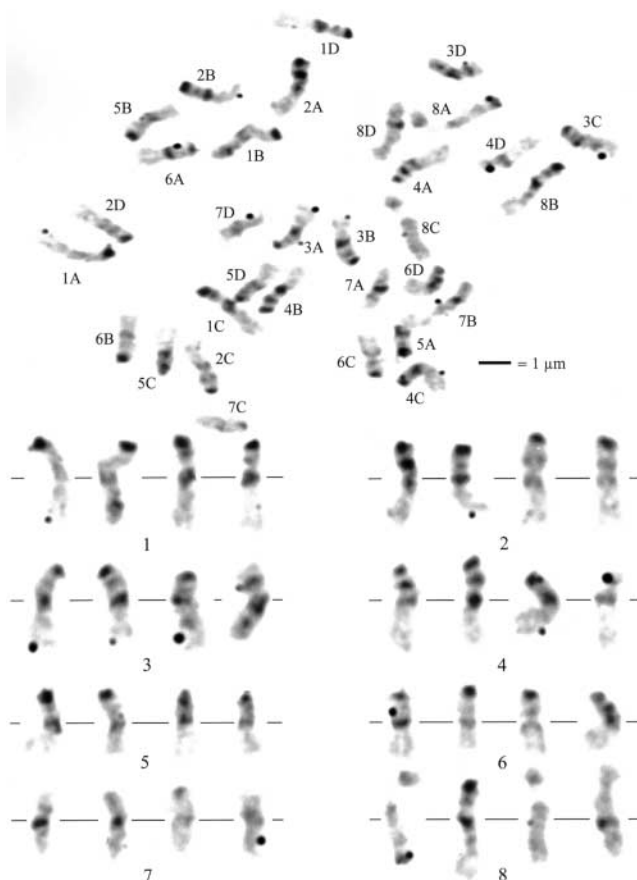


Fig. 1. C-banded karyotype of *M. sativa* subsp. *sativa*. Dots on the chromosomes do not represent a C-band but are artifacts of preparation. The bar represents one micrometer

pattern. None of the genotypes evaluated in this and earlier studies (Bauchan and Hossain, 1998b) has had as many heterochromatic bands as those found in the African germplasm source. Polymorphisms in C-banded constitutive heterochromatic DNA were found among individuals in the Chilean germplasm source. The abundant polymorphisms are not surprising, considering the out-crossing nature of alfalfa.

The efficacy of the image analysis system for discriminating among chromosome sets on the basis of Tukey's test (SAS, 1999) as determined by this study and previous studies (Bauchan and Campbell, 1994; Bauchan and Hossain, 2001a,b) showed that the only measurement which could be used reliably to distinguish the chromosomes was relative chromosome length (Table 1). The coefficients of variation (CV) indicates that parameter estimation was reasonably precise.

Chromosome Descriptions and Comparison to the Reference Karyotype

Chromosome 1. The largest chromosome with an average length of 2.74 μm (Table 1) without a nucleolar 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. The interstitial bands on the long arms of the chromosomes were absent in two plants, one plant was missing the band on one homolog, and the other plant lacked the band on two homologs. There were two different individuals which had lost interstitial bands on the short arms of the chromosomes, one plant lacked the band on one homolog and the other plant was missing the band on two homologs. One plant had an extra interstitial band on the long arm. Compared with the reference African karyotype this Chilean chromosome was occasionally missing the interstitial band on the short arms, whereas all of the African plants studied had interstitial bands on the short arms (Fig. 2).

Chromosome 2. A submetacentric chromosome with a large telomeric band on the short arm and an interstitial band located on each arm of the chromosome. The interstitial bands on the long arms on all four homologs were missing in 31% of the plants studied. One plant was missing the interstitial band on two of the homologs.

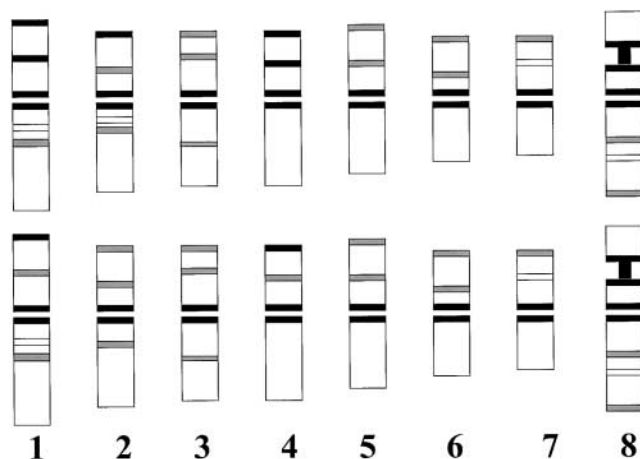


Fig. 2. Summary idiograms of the polymorphisms which were observed in the C-banded chromosomes of *M. sativa* subsp. *sativa* African (top) and Chilean (bottom). Solid bands represent bands which were always present in all four homologs. Shaded bands represent bands which were not always present in all four homologs. Open bands represent bands which occasionally were present (<4%).

The interstitial bands were absent on the short arms of three plants, in two plants all four homologs were lacking, and in one plant only two homologs had lost the band. One homolog on one plant was missing the terminal band on the short arm. The major differences between the reference karyotype and the Chilean karyotype are the lack of an occasional second interstitial band on the long arm, the rare deletion of a terminal band and the reduction in the frequency of the appearance of interstitial bands on the long arm.

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 are not as prominent as the one 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 Chilean alfalfa genome. The most frequent polymorphism (68%) was the loss of the interstitial bands on the long arms. Seven of the 19 plants studied (37%) were missing bands on all four homologs, 21% were lacking bands on three homologs, and 26% were lacking bands on either one or two homologs. The interstitial bands on the short arms were also absent in

Table 1. Efficacy of the image analysis system for differentiating among homologous chromosome sets in Chilean alfalfa for 17 different plants.

Chromosome set	Average short arm	Average long arm	Arm ratio	Average total length	Relative length (%)†	Average SAT length
1	1.00 ± 0.02a‡	1.43 ± 0.03a	1.43 ± 0.01b	2.43 ± 0.03b	13.83 ± 0.03b	
2	0.96 ± 0.02a	1.32 ± 0.02b	1.38 ± 0.01b	2.28 ± 0.02c	12.98 ± 0.03c	
3	0.95 ± 0.03a	1.26 ± 0.02c	1.33 ± 0.01b,c	2.21 ± 0.03d	12.58 ± 0.02d	
4	0.92 ± 0.03a,b	1.20 ± 0.02c	1.30 ± 0.01b,c	2.12 ± 0.03e	12.07 ± 0.02e	
5	0.90 ± 0.03a,b	1.16 ± 0.03c,d	1.29 ± 0.01b,c	2.06 ± 0.03e	11.72 ± 0.02f	
6	0.86 ± 0.02b,c	1.10 ± 0.03d,e	1.28 ± 0.01c	1.96 ± 0.03f	11.16 ± 0.03g	
7	0.83 ± 0.03b,c	1.00 ± 0.02e	1.20 ± 0.01c	1.83 ± 0.02f	10.42 ± 0.03h	
8	0.81 ± 0.03c	1.33 ± 0.03b	1.64 ± 0.01a	2.74 ± 0.03a	15.59 ± 0.02a	0.71 ± 0.03
CV§	9.33	9.52	6.70	9.02	7.85	

† Relative length as a percent of total length of all eight chromosomes.

‡ Means in the same column followed by different letters are significantly different at $P < 0.05$ based on Tukey's test. Measurements are in micrometers followed by standard deviation (\pm).

§CV = Coefficient of variation.

six individuals, two plants were missing interstitial bands on all four homologs, two plants were lacking bands on three of the homologs, and the interstitial band was deficient on one homolog in the other two plants. Occasionally (26%), terminal bands were deleted on the short arms. Chromosomes 3 in the reference karyotype and the Chilean karyotype are very similar; however, the number of bands absent in the Chilean karyotype is 8% greater than was found in the African reference karyotype.

Chromosome 4. A submetacentric chromosome with an interstitial band midway between the telomeric band and the centromeric band; there were no interstitial bands located on the long arms, although occasionally a tertiary constriction can be found on the long arms of the chromosomes. This chromosome had a highly conserved banding pattern as was the case for the reference karyotype. In all but one of the plants studied, all of the homologs had terminal bands and interstitial bands on the short arms with the exception of one plant which was missing the interstitial band on two homologs.

Chromosome 5. A metacentric chromosome with an interstitial band closer to the centromeric band than to the telomeric band on the short arm. The most prevalent deviation, occurring in 5 of the 19 (26%) plants studied, was the loss of the interstitial bands on the short arms. One plant each was missing bands on one, two, or three homologous chromosomes, while in the other two plants bands were absent on all four homologs. Only one individual was observed to be lacking terminal bands and that occurred on two of the four homologs. There are only slight differences between the reference karyotype and the Chilean karyotype. Compared to the African homologs there were 5% fewer interstitial bands observed on the short arms of Chromosome 5 in all the Chilean homologs.

Chromosome 6. Another metacentric chromosome with terminal bands and interstitial bands on the short arms. In almost half (47%) of the plants, the interstitial bands were lost on all four of the homologs. There were two plants which were observed that were lacking the terminal band on the short arms. The absence of interstitial bands on the short arms in the Chilean karyotype is one of the major differences between the Chilean and African genomes.

Chromosome 7. The shortest chromosome in the genome [an average length of 1.83 μm (Table 1)], a metacentric chromosome with only centromeric bands and telomeric bands on the short arms of the chromosome with occasional (5%) interstitial bands on the short arms. Two of 19 plants were missing the terminal band on one of the homologs on the short arm. This Chilean chromosome is again very much like the reference karyotype; however, it appears to have retained more terminal bands than were observed in the African reference karyotype. Chilean chromosomes were only missing the terminal band in 2 of the 19 plants observed, whereas 8 of the 25 African chromosomes were lacking the terminal band.

Chromosome 8. The only satellited chromosome (SAT), submetacentric 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 chromosomes. A majority (84%) of the plants were polymorphic for this chromosome. The interstitial bands on the long arms were missing on 63% of the homologs. Forty-two percent were lacking the band on one of the homologs and 21% of them were deficient for the bands on two of the homologs. In three of the 19 plants a double interstitial band occurred on the long arms. Occasionally (16%), the terminal bands were missing on the long arms of the chromosomes. Chromosome 8 in both the Chilean and African germplasm sources were highly polymorphic. Figure 2 is a composite idiogram for the Chilean germplasm source compared to the reference African germplasm source.

Abundant variability was noted in the number, intensity, and location of the constitutive heterochromatic DNA in Chilean alfalfa. The additional bands could have preexisted, resulted from reduplication of highly repetitive DNA, or could have been the result of unequal crossing over or a translocation (Stanford and Clement, 1958). The loss of a terminal band can occur through a deletion or possibly through outcrossing with *M. sativa* subsp. *falcata*. Bauchan and Hossain (1997, 1999a) demonstrated that diploid *M. sativa* subsp. *falcata* chromosomes possess bands primarily at the centromeres. A preliminary study of tetraploid *M. sativa* subsp. *falcata* chromosomes revealed a larger number of C-bands than had been noted in diploid *M. sativa* subsp. *falcata*; however, there were fewer bands than had been noted in *M. sativa* subsp. *sativa* (Bauchan and Hossain, 1998b, 1999b). Hybridization between *M. sativa* subsp. *falcata* and *M. sativa* subsp. *sativa* can occur naturally, and meiotic crossing-over can result in the loss of constitutive heterochromatic DNA. Selection towards the reduction of heterochromatin is favored. The nondormant Chilean germplasm source appears to contain primarily subsp. *sativa* germplasm, as expressed in the consistency with which all four homologs had bands at the same location. In preliminary cytogenetic studies of the nine germplasm sources, Bauchan and Hossain (1998b) found that the more fall dormant germplasm sources contained a lesser amount of constitutive heterochromatic DNA.

Five of the 50 total Chilean clones studied were aneuploids, one trisomic ($2n = 4x + 1 = 33$) and four monosomics ($2n = 4x - 1 = 31$). These plants were each karyotyped from two well spread cells and it was noted that aneuploidy was due to an extra or missing SAT chromosome. Aneuploids ranging in chromosome number from $2n = 30$ to 35 have been discovered in 'Vernal' and 'Saranac' alfalfa (Bingham, 1968) and trisomics at the diploid ($2n = 2x + 1 = 17$) and tetraploid level are easily recovered following triploid \times diploid and triploid \times tetraploid crosses, respectively (Stanford, 1959; Kasha and McLennan, 1967; Buss and Cleveland, 1971; Binek and Bingham, 1970; and reviewed by McCoy and Bingham, 1988). Schlarbaum et al. (1988) discovered an aneuploid cultured from a single protoplast that was missing a SAT chromosome. Aneuploids could have arisen through two possible mechanisms: (i) non-

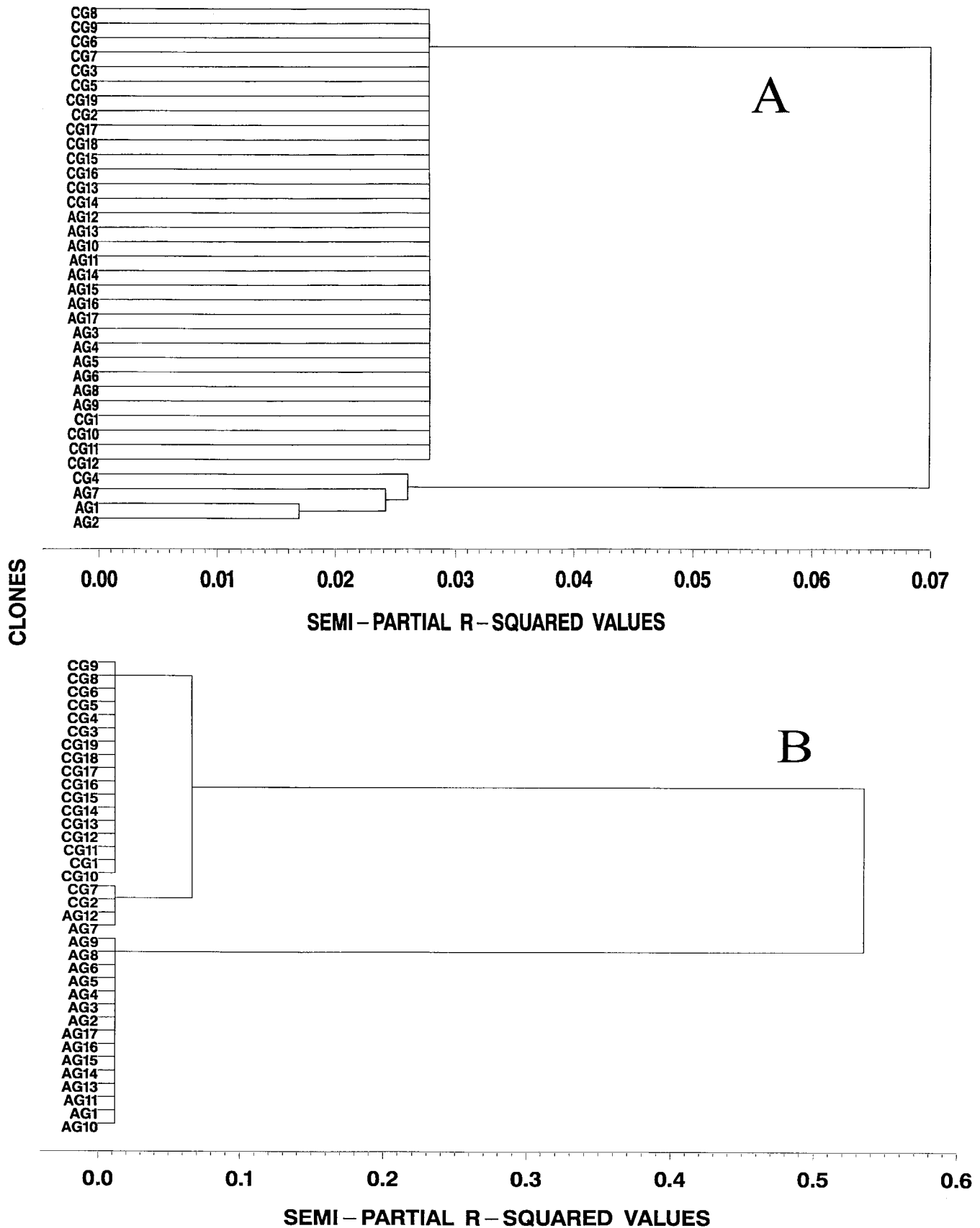


Fig. 3. Dendrogram from Ward's minimum variance cluster analysis of genetic distances among 36 alfalfa clones tracing to Chilean (CG) or African (AG) germplasm sources. Dendrograms based on chromosomal C-banding patterns of Homolog 1 of all chromosomes (A) and of Chromosome 8 only (B) are presented.

Table 2. Percentages of the time identical clusters were derived during a Ward's minimum variance bootstrap cluster analysis (100 cycles) of genetic distances among 36 alfalfa clones tracing to Chilean or African germplasm sources.

Cluster No.	Percentage of time identical clusters were derived
1	30
2	12
3	12
4	12
5	16
6	16
7	16
8	16
9	16
10	16
11	16
12	12
13	12
14	12

disjunction of quadrivalents (Cleveland and Stanford, 1959; Grun, 1951; McLennan et al., 1966) and/or univalents and trivalents (Julen 1944; Cleveland and Stanford, 1959; Grun, 1951; McLennan et al., 1966) or (ii) asynaptic plants, which occasionally occur in diploid alfalfa (Bolton and Greenshields, 1950; Bingham and Gillies, 1971) and can have unequally distributed chromosomes.

Statistical Analyses of C-Banding Patterns

Cluster analysis based on all eight chromosomes yielded no clear separation of Chilean and African

germplasm (Fig. 3A); however, an analysis of the highly polymorphic Chromosome 8 was fairly effective in separating the two germplasm sources (Fig. 3B), although genotypes CG2, CG7, AG7, and AG12 were clustered together. As a matter of interest, it was noted that GDs based on a simple matching coefficient yielded the same clusters. Bootstrap analysis of clustering based on Chromosome 8 data (Table 2) indicates that cluster repeatability was quite low. Such results are often indicative of a weak hierarchical data structure. Local maxima for the cubic clustering criterion (CCC) and the pseudo *F*-value (SAS Institute, 1999) are often associated with a reasonable estimate of the true number of population clusters. Neither statistic reached a local maximum in the analysis, and these results appear to verify the weak hierarchical structure of the data. Multiple correspondence analysis of Chromosome 8 data was more definitive and placed African and Chilean entries in five separate clusters (Fig. 4). Genotypes CG2 and CG7 were placed in a separate cluster by this analysis whereas they were clustered with AG12 and AG7 by Ward's analysis. It is possible that other homologs of this or other chromosomes may also separate the two germplasm sources effectively.

This study has shown that through the combined use of C-banding and image analysis techniques it is possible to identify individual alfalfa chromosomes for comparison with a reference karyotype, for assessing germplasm diversity, and for the identification of aneuploids. The

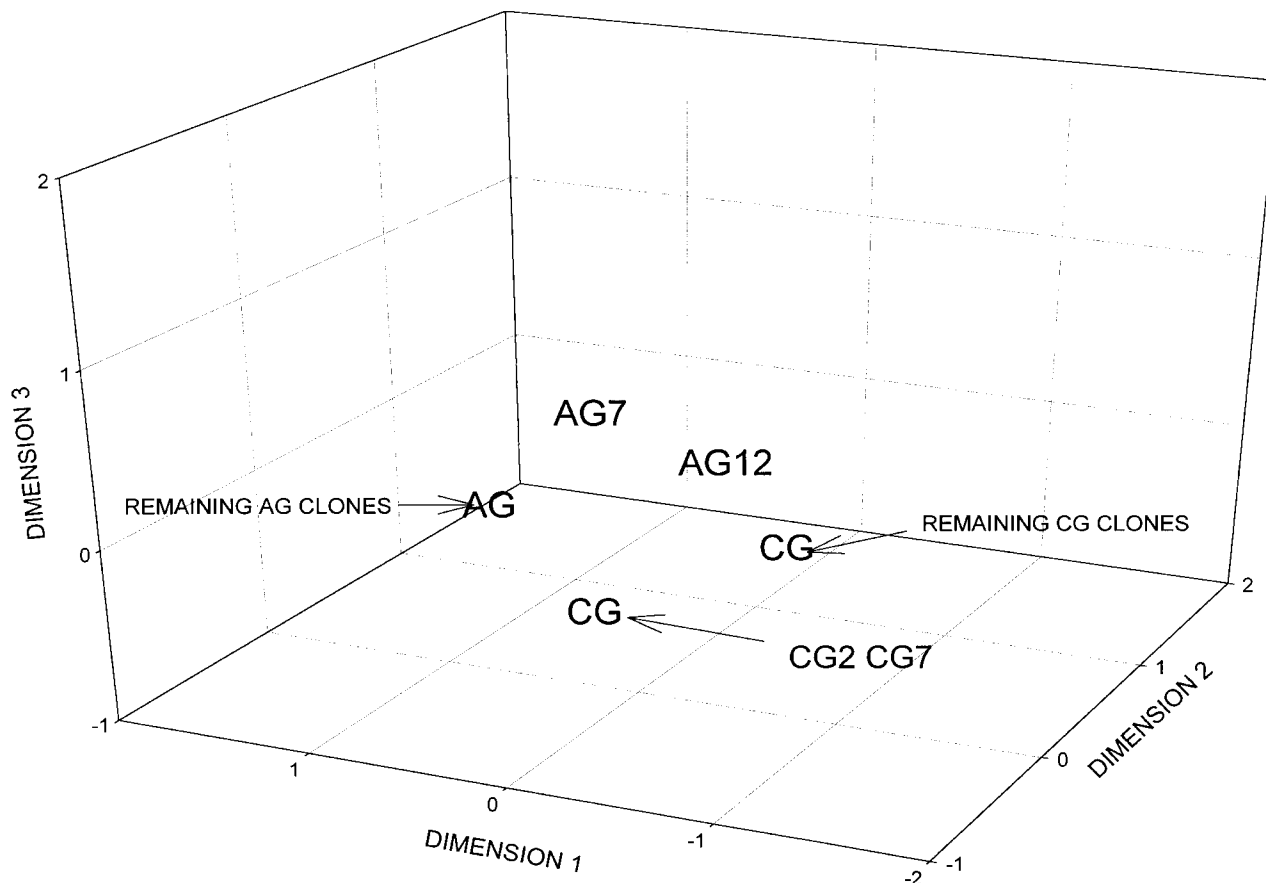


Fig. 4. Multiple correspondence analysis of genetic distances, based on C-banding patterns of Chromosome 8 Homolog 1, among 36 alfalfa clones tracing to Chilean (CG) or African (AG) germplasm sources.

cluster and multiple correspondence analysis demonstrated that C-band polymorphisms can be fairly effective in distinguishing karyotypes of different germplasm sources.

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