Comparative Chromosome Banding Studies of Nondormant Alfalfa Germplasm

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ABSTRACT

A cytogenetic investigation was conducted on four historically putatively distinct nondormant alfalfa germplasm sources, African, Chilean, Peruvian, and Indian tetraploid alfalfa [*Medicago sativa* ssp. *sativa* (L.) L. & L.; $2n = 4x = 32$]. C-banding, image analysis, and cluster analysis was used to test the hypothesis that chromosome structure differed among the four nondormant alfalfa populations. Cytogenetic analyses revealed polymorphisms for heterochromatic DNA in the number and location of constitutive heterochromatic DNA both within and among genotypes. However, this variability did not prevent recognition of homologous chromosomes. Karyotypes of Peruvian and Indian populations were developed. The reference African population was used to compare the karyotypes of Peruvian and Indian populations as well as the previously published Chilean population. In general, the number of heterochromatic DNA bands was similar for the African, Chilean, and Peruvian populations; however, the Indian population had significantly fewer heterochromatic bands than the other three. Cluster analysis based on all eight chromosomes yielded no clear separation of the nondormant alfalfa populations possibly because of the lack of chromosomal rearrangements, similar genetic backgrounds of the initial introductions, intercrossing of the different sources, genetic drift during maintenance, and/or common genetic backgrounds of the original parental germplasm sources.

Alfalfa, the most important forage crop grown in the USA, is primarily harvested as hay for animal consumption but is also an important component of pastures. Nondormant alfalfa is particularly important in the southwestern USA where, under irrigation, it can be grown throughout the year producing 52 Mg ha^{-1} yr^{-1} compared with 22 Mg ha^{-1} yr^{-1} for alfalfa grown under varied conditions in the midwestern USA. Nondormant alfalfa is characterized as having rapid regrowth after harvesting in the autumn but it is not winter hardy (Barnes et al., 1978; Teuber et al., 1998).

Nine historically distinct sources of alfalfa germplasm, African, Chilean, Flemish, Indian, Ladak, Peruvian, Turkistan, *M. falcata*, and *M. varia*, were introduced into different U.S. regions between 1850 and 1947 (Barnes et al., 1977). Nondormant alfalfa was introduced into the new world initially by the Spanish as early as the 16th Century. The Chilean and Peruvian types spread into the southwestern USA with missionaries as early as 1850. The Indian types were introduced into California in 1913 (Barnes et al., 1977). Melton et al. (1990) developed broad-based populations of the nine historical germplasm sources.

Several cytogenetic studies have been conducted to karyotype the somatic chromosomes of tetraploid alfalfa (Agarwal and Gupta, 1983; Falistocco, 1987; Schlarbaum et al., 1988; Falistocco et al., 1995; Bauchan and Hossain, 2001a,b; Bauchan et al., 2002). The alfalfa karyotype consists 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). Chromosome banding studies have identified individual chromosomes of *M. sativa* subspecies, including *coerulea, falcata* (Bauchan and Hossain, 1997, 1998a, 1999a,b,c) and *sativa* (Falistocco et al., 1995; Bauchan and Hossain, 2001a,b; Bauchan et al., 2002). Bauchan and Hossain (2001b) evaluated the African tetraploid population and concluded it resembled the C-banding pattern of diploid *M. sativa* subsp. *coerulea* (Bauchan and Hossain, 1997). The African germplasm source revealed the most heterochromatic DNA of the nine alfalfa germplasm sources (Bauchan and Hossain, 1998b) and its C-banded karyotype was used as the reference karyotype for characterizing the Chilean population (Bauchan et al., 2002).

The objective of this experiment was to test the hypothesis that the four nondormant alfalfa germplasms introduced into the USA between 1850 and 1950 have different chromosomal structures as defined by C-banding patterns.

MATERIALS AND METHODS

Karyotype Analyses

Seeds of the Indian and Peruvian populations were obtained from the U.S. Plant Introduction Station at Pullman, WA. The registration number, plant inventory number, and parental germplasm sources are listed for the four populations evaluated in this experiment (Table 1). Twenty plants of each population were grown in the greenhouse to determine if the seed source was true to form for growth habit, flower color, and pod type. Seeds were scarified and germinated in Petri dishes at 25°C on filter paper. Root tips (5–10 mm in length) were collected 2 to 3 d after germination, pretreated in an ice bath (0°C) 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) on 10 cells in each of 20 Peruvian and 20 Indian individual plants. Sufficiently spread C-banded chromosomes from each germplasm population were karyotyped. Chromosomes were observed 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). Karyotypic analyses were conducted 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). By convention, homologs were aligned within a set

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Table 1. Alfalfa population registration number, plant inventory number, and parental germplasm sources (USDA National Plant Germplasm System, 2002).

Population	Registration number	PI number	Parental sources
African	GP-238	536539	'Lew', 'Sonora', 'Sonora 70', Bryan Syn., African 4-31-A, 'Moapa', African 4-44-A
Peruvian	GP-234	536535	'Coastal Hairy Peruvian', 'Hairy Peruvian'
Chilean	GP-233	536534	'Calivarde', 'California Common', 'California Common 49', 'Chilean Common', Chilean 22-5, 'Chilean 21-5-5'
Indian	GP-235	536536	'Sirsa No. 9', 'Arizona Indian', 'Mesa Sirsa'

starting with the chromosome with the greatest number of heterochromatic bands to the least. The karyotypes of the African (Bauchan and Hossain, 2001b) and Chilean (Bauchan et al., 2002) populations were developed by the same procedure.

The efficacy of the image analysis system to differentiate among alfalfa chromosomes was tested on all four germplasm sources. There were no significant differences among the data of the four germplasm sources; thus, the data were combined. Differences in gross chromosomal morphology and mean number of heterochromatic bands were tested among chromosomes and among germplasm sources by Tukey's test in the SAS statistical software package (SAS, 1999). Adjustments to the karyotype were made by swapping chromosomes between homologous groups if a chromosome was distorted during the squashing process and/or if the original classification disagreed with banding patterns. For comparison purposes, idiograms were developed for each plant that displayed polymorphisms. Figure 1 was produced by means of Adobe Photoshop (Adobe Systems, Inc., San Jose, CA).

Statistical Analyses of C-Banding Patterns

Cluster analysis was used to determine if C-banding patterns could be used to differentiate the four nondormant germplasm sources. Phenotypes of homolog 1, the homolog with the greatest number of heterochromatic bands, were clustered. For each chromosome, all genotypes were scored for presence or absence of C-bands, from which a 1/0 matrix was developed (Bauchan and Hossain, 2001b; Bauchan et al., 2002).

Genetic distances (GDs), based on all pairwise comparisons for the eight chromosomes together and for each chromosome separately, were computed by the following 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, respectively (Nei and Li, 1979). This formula disregards 0/0

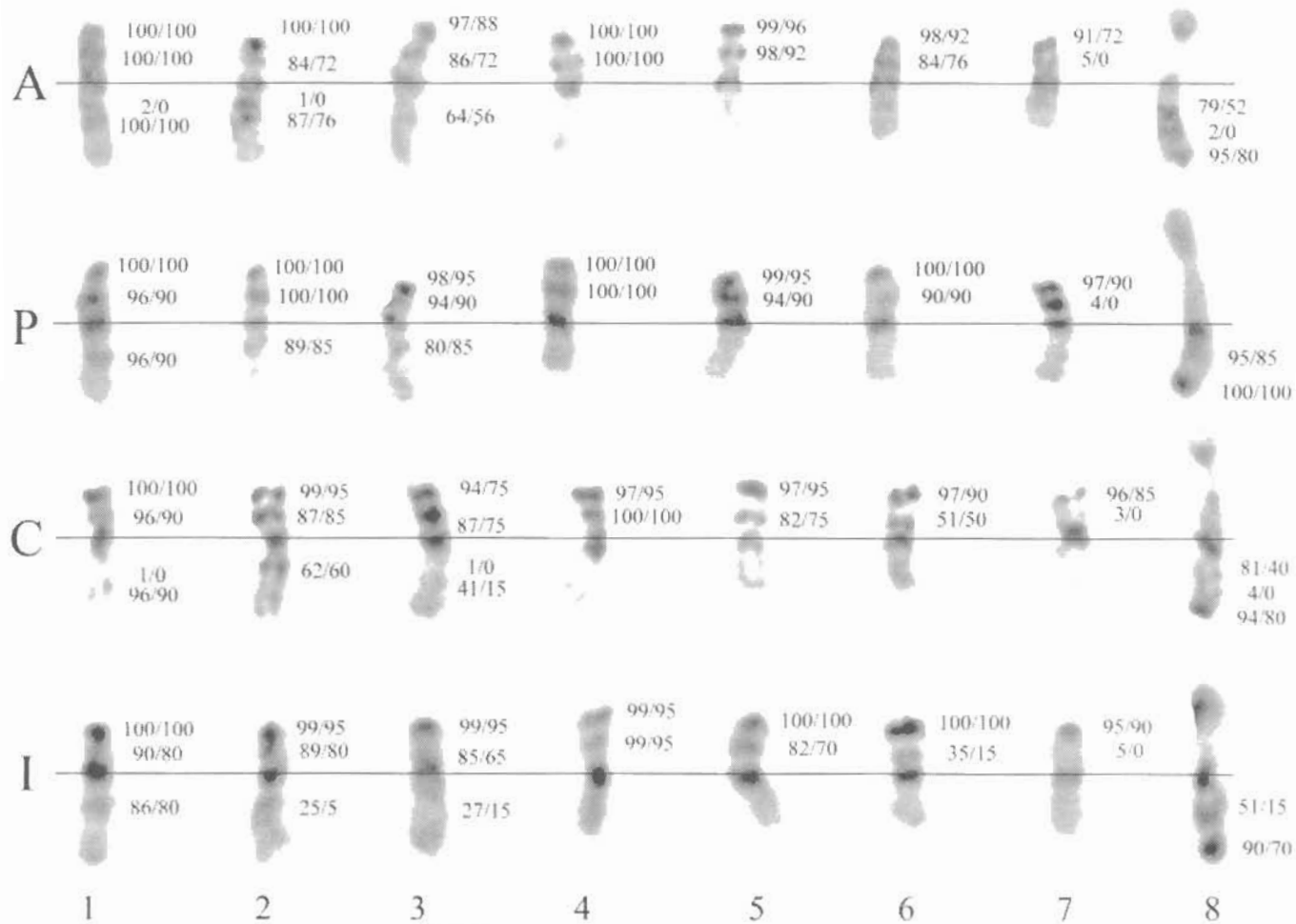

Fig. 1. Comparison of the C-banding patterns of African, Peruvian, Chilean, and Indian alfalfa populations. The first number next to each band is the percentage of homologous chromosomes possessing the heterochromatic bands. The second number is the percentage of plants that had chromosomes with heterochromatic bands on all four homologs.

Table 2. Efficacy of the image analysis system for differentiating among homologous chromosome sets averaged across four nondormant alfalfa germplasm populations. Measurements are in micrometers followed by standard error.

Chromosome set	Average short arm	Average long arm	Arm ratio	Average total length	Relative length	Average SAT length
					%†	
1	1.00 ± 0.02a‡	1.44 ± 0.03a	1.44 ± 0.01b	2.44 ± 0.03b	13.70 ± 0.03b	
2	0.98 ± 0.02a	1.31 ± 0.02b	1.37 ± 0.01b	2.29 ± 0.02c	12.86 ± 0.03c	
3	0.97 ± 0.03a	1.25 ± 0.02c	1.29 ± 0.01b,c	2.22 ± 0.03d	12.46 ± 0.02d	
4	0.94 ± 0.03a,b	1.20 ± 0.02c	1.28 ± 0.01b,c	2.14 ± 0.03e	12.02 ± 0.02e	
5	0.92 ± 0.03a,b	1.14 ± 0.03c,d	1.24 ± 0.01b,c	2.06 ± 0.03e	11.57 ± 0.02f	
6	0.87 ± 0.02b,c	1.09 ± 0.03d,e	1.25 ± 0.01c	1.96 ± 0.03f	11.01 ± 0.03g	
7	0.85 ± 0.03b,c	1.03 ± 0.02e	1.21 ± 0.01c	1.88 ± 0.02f	10.56 ± 0.03h	
8	0.82 ± 0.03c	1.34 ± 0.03b	1.63 ± 0.01a	2.82 ± 0.03a	15.83 ± 0.02a	0.66 ± 0.03
CV	9.67	10.52	6.70	9.20	7.65	

† Relative length as a percentage of total length of all eight chromosomes.
‡ Means in the same column followed by different letters are significantly different at $P < 0.05$ on the basis of Tukey's test.

matches, which could be caused by factors other than genetic similarity, and weights 1/1 matches by a factor of 2 to better separate genotypes.

Genotypes were clustered by Ward's Minimum Variance hierarchical cluster analysis (PROC CLUSTER, SAS Inst., 1999), where the distance between two clusters is the analysis of variance sum of squares between the clusters summed over all variables. For interpretation, the sums of squares were 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. Genetic distances were squared before invoking the procedure. Dendrograms were produced by PROC TREE and enhanced by PROC GPLOT (SAS Inst., 1999).

RESULTS AND DISCUSSION

Observations of the growth habit (upright), flower color (purple), and pod type (curled) of 20 plants from the Indian and Peruvian population grown in the greenhouse were judged to fit the alfalfa phenotype for each respective population. Enhancement of the chromosomal images by pseudocoloration (by which a color enhanced image is produced by taking a densitometric measurement of the image and assigning each pixel a color) and digital enlargement enabled the edges of the chromosomes and the heterochromatic bands to be distinguished for identification and measurement. All of the cells within an individual plant had the same banding pattern. Because the African germplasm population karyotype (Bauchan and Hossain, 2001b) had more heterochromatic bands than any of the other genotypes evaluated in this and earlier studies, it was used as a reference for comparison purposes. Polymorphisms in constitutive heterochromatic DNA were found among individuals in all four germplasm sources. The abundant polymorphisms were not surprising, considering the outcrossing nature of alfalfa. The karyotypic comparative analysis of the four germplasm types was done with 20 plants from each of the four populations. The Peruvian and Indian karyotype were produced in this study and the African (Bauchan et al., 2001b) and Chilean (Bauchan et al., 2002) karyotypes were produced in previous studies.

Because no differences of chromosomal morphometric data were observed among the four germplasm sources, the data were combined (Table 2). The only measurement which could be used reliably to distinguish among the chromosomes was relative chromosome length (Table 2), which confirms previous results (Bauchan and Campbell, 1994; Bauchan and Hossain 2001a,b; Bauchan et al., 2002). The coefficients of variation indicates that parameter estimation was reasonably precise when the image analysis system was used.

The main differences between the African and Peruvian karyotype are the lack of an occasional double band on the long arm of chromosome 1 and the absence of two interstitial bands on the long arm of chromosome 8 (Fig. 1). The major differences between the Indian and African germplasm are that Indian lacks an occasional double band on the long arm of chromosomes 1 and 2, has fewer interstitial bands on the long arm of chromosome 3, has only a rare (1%) occurrence of an interstitial band on the short arm of chromosome 7, and has the deficiency of a double interstitial band on the long arm of chromosome 8. The Indian germplasm population had fewer ($P < 0.05$) heterochromatic DNA bands than the other three nondormant germplasm sources (Table 3). Comparison of the Chilean karyotype to the reference African karyotype has been detailed previously (Bauchan et al., 2002).

Table 3. Total number of heterochromatic DNA telomeric or interstitial bands per plant with mean for each population.

Plant number	African	Peruvian	Chilean	Indian
1	72	70	72	67
2	72	70	72	66
3	72	69	71	64
4	72	69	69	63
5	71	69	69	63
6	70	69	68	61
7	69	68	67	59
8	69	68	66	59
9	68	67	65	59
10	66	66	64	58
11	65	65	64	58
12	63	63	64	58
13	63	63	59	57
14	62	62	58	57
15	62	62	58	55
16	61	61	57	54
17	61	61	56	52
18	59	59	54	51
19	59	57	53	50
20	59	51	52	50
Mean†	65.8a	64.5a	62.9a	58.1b

† Mean followed by different letters are significantly different at $P < 0.05$ on the basis of Tukey's means separation (SAS, 1999).

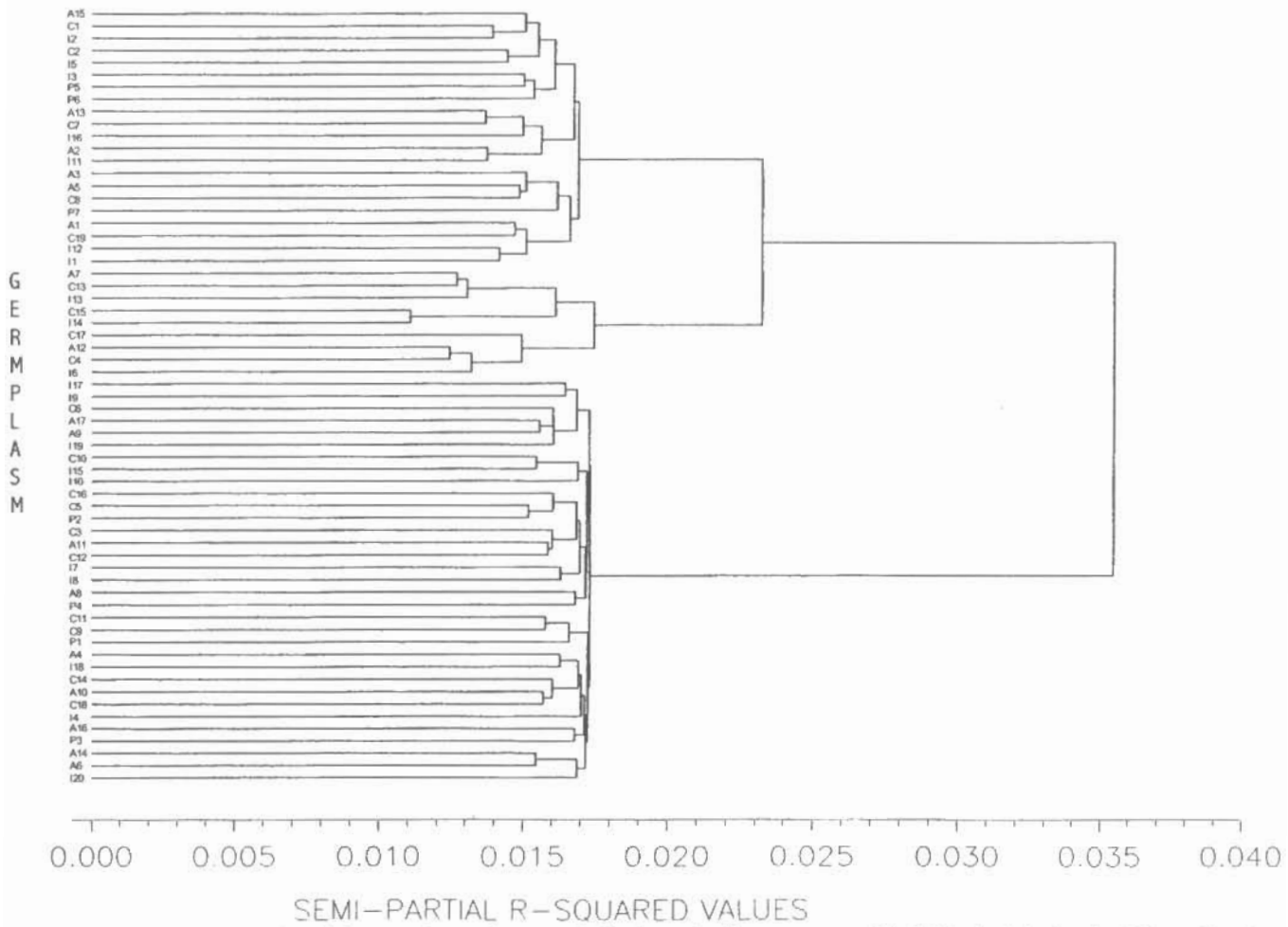

Fig. 2. Dendrogram from Ward's minimum variance cluster analysis of genetic distances among 80 alfalfa plants tracing to African, Peruvian, Chilean, and Indian alfalfa populations. Dendrograms are based on the chromosomal C-banding patterns of all eight chromosomes.

Comparison of All Four Nondormant Alfalfa Populations

Abundant variability was noted in the number and location of the constitutive heterochromatic DNA in nondormant alfalfa. These could have preexisted the populations formation, resulted from reduplication of highly repetitive DNA (Bickmore and Craig, 1997), or been the result of unequal crossing over or a translocation (Stanford and Clement, 1958). The loss of a terminal band could occur through a deletion or possibly through outcrossing with *M. sativa* ssp. *falcata*, which Bauchan and Hossain (1997, 1999a) demonstrated possesses primary centromeric bands. Meiotic crossing-over in *sativa–falcata* subspecies hybrids could lead to the loss of constitutive heterochromatic DNA. The nondormant germplasm sources appear to contain primarily subsp. *sativa* germplasm, as expressed in the consistency with which all four homologs had bands at the same location.

Cluster analysis based on all eight chromosomes yielded no clear separation of the four nondormant populations, indicative of a weak hierarchical data structure (Fig. 2). However, the Indian germplasm did have significantly fewer heterochromatic DNA bands than the other three nondormant germplasm sources. Kidwell et al. (1994) used RFLP (restriction fragment length polymorphisms) markers and Segovia-Lerma et al. (2003) used AFLP (amplified fragment length polymorphisms) markers to analyze these same nine germplasm types. Kidwell et al. (1994) found that the Falcata and Peruvian germplasms could be distinguished from the other germplasms and Segovia-Lerma et al. (2003) identified two main clusters: *M. sativa* subsp. *sativa* and *M. sativa* subsp. *falcata*. Falcata is easily distinguished morphologically by its yellow flower color, sickle shaped pods, and prostrate growth habit (Small and Jomphe, 1989). Peruvian can sometimes be identified morphologically by its abundant simple hairs, especially with "Hairy Peruvian types" (Barnes et al., 1977). The Peruvian germplasm type may be unique because it was developed from the selection of the Spanish types initially brought into the New World and grown in Peru and only two original parental germplasm sources were used to develop the population evaluated in this study, whereas the other broad-based populations contained from three to 10 original parental germplasm sources (Melton et al., 1990). Thus, the phenotypic differences among the four nondormant germplasm types may not reflect large changes in chromosome structure.

Alfalfa is an out-crossing polyploid species that is composed of highly heterogeneous populations. Therefore, as much variation can be found within populations as between populations (Kidwell et al., 1994; Ray et al., 1998). However, if the four populations studied here actually represent distinct nondormant germplasm sources, there should be better separation between populations. A number of explanations can account for these results. First, the original germplasm sources may not have been very distinct when introduced into the USA. Although alfalfa was first cultivated nearly 9000 yr ago (Ivanov, 1977), most of the distribution throughout the world from southeastern Asia has occurred within the past 500 yr (Michaud et al., 1988). The initial populations were heterogenous and intercrossing, and thus may not have undergone major selective changes, or if they did, selections only affected a small subset of the loci before introduction into the USA. Second, the four germplasm sources may not faithfully represent the original germplasm sources because of outcrossing, selection, and genetic drift during seed increase. After the germplasm was introduced into the USA, it was maintained in different collections, many of which used open-pollination for seed increase and maintenance (Barnes et al., 1977). Thus, out-crossing would have occurred to homogenize the various germplasm sources. Third, the populations had some common ancestral backgrounds. An example is the cultivar Lew, one of the parental African sources, the pedigree of which includes both African and Indian germplasm sources (USDA, National Plant Germplasm System, 2002).

This study used C-banding and image analysis techniques to assess germplasm diversity in chromosomal structure among four nondormant alfalfa germplasms; the inability to differentiate among three of the four nondormant types of alfalfa in this study is consistent with that observed previously (Kidwell et al., 1994; Ray et al., 1998; Segovia-Lerma et al., 2003) and could reflect similar genetic backgrounds of the initial introductions of the four germplasm into the USA, intercrossing of the different sources and/or genetic drift during maintenance, or common genetic backgrounds of the parental sources used by Melton et al. (1990). By analyzing the mean number of C-banded chromosomes, the Indian germplasm can be identified by the reduced number of heterochromatic DNA bands. We recommended that future studies of the nine historically distinct alfalfa sources should include the original parental germplasm sources used by Melton et al. (1990) rather than the subsequent broad-based populations developed in that study.

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