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Non random chloroplast DNA hypervariability in *Medicago sativa*

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Abstract Two hypervariable regions of the alfalfa (*Medicago sativa* L) chloroplast genome were used to describe levels of genetic relatedness among populations. PCR primers were developed to amplify the hypervariable regions. The frequency of occurrence of fragments of like size between populations was used to develop a measure of genetic relatedness. Relationships among 135 alfalfa accessions were investigated with principal component and cluster analyses, based on the genetic distance measures. Distinct clusters were taken as an indication of genetically distinct lineages. The populations investigated represented collections from world regions defined as the centers of origin of specific alfalfa germplasm sources, or else represented collections of introduced, and naturally adapted, accessions from agriculturally advanced regions. In general, this analysis indicated that the accessions from regions of origin of germplasm sources were largely homogeneous, while accessions from areas of introduction were much more diverse. In some cases, the accessions from a region of origin formed distinct clusters, suggesting that divergence has resulted in genetically distinct lines persisting in the original region of origin. Investigation of the stability of the marker fragments through vegetatively, and sexually, propagated plants indicated stable transmission through the sexual phase. However, one of the two regions underwent a deletion of 145 bp of one copy of a tandemly repeated 146 bp region in the equivalent of 80 years of vegetative growth.

Keywords *Medicago · Alfalfa · Lucerne · Diversity · DNA sequence · cpDNA · Cluster analysis · AF237706 · AF237707*

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Introduction

The coding regions of chloroplast genomes of higher plants are highly conserved across species and genera. However, some regions of the chloroplast genome have been described as highly variable in several species. Ogihara et al. (1988, 1991, 1992) identified a "hot spot" for length mutations in wheat chloroplast DNAs. Provan et al. (1996) used a hypervariable microsatellite-like region of the rice chloroplast genome to derive DNA fingerprints of 67 rice accessions; 46 unique haplotypes were found. The occurrence of the 46 haplotypes was not random but, rather, reflected geographic or subspecies differentiation. Also using microsatellite-like regions, hypervariability was described in the chloroplast DNA of *Glycine* species (Powell et al. 1996) and *Pinus* species (Powell et al. 1995). Vendramin et al. (1998) also examined hypervariable regions of various coniferous tree species and used haplotype variation to distinguish populations.

In alfalfa (*Medicago sativa* L), Johnson and Palmer (1989) identified individual alfalfa plants which appeared to have multiple forms of the chloroplast DNA molecule (heteroplasmic plants), and also described a genomic region which was hypervariable between *Medicago* species. Schabel (1996) reported a short hypervariable region of the alfalfa chloroplast DNA that showed extreme variation within the *M. sativa* species. Both of these studies (Johnson and Palmer 1989; Schabel 1995) used RFLP analysis to observe the variation. Neither report investigated the variability within and among *M. sativa* populations.

There currently are thought to be ten basic origins of cultivated alfalfa (Barnes et al. 1977; Smith 1991). Populations from these basic origins were described on the basis of morphological or physiological traits (Barnes et al. 1977; Smith 1991). Historically, alfalfa cultivars and germplasms have also been distinguished by physiological or morphological measurements, often involving measurements of disease or insect resistance. However, plant collections from very diverse origins often cannot be distinguished by any of the morphological measure-

ments. Molecular markers provide a potential means to distinguish alfalfa lines. However, random markers such as RFLPs or PCR-based markers from random amplifications (RAPDs, AFLPs, etc.) have proven to be highly variable such that distances within populations often cannot be distinguished from variation between populations (Kidwell et al. 1994). The objectives of the present study were to develop specific PCR primers and procedures to detect variation in the chloroplast DNA hypervariable regions, to use fragment length polymorphism in hypervariable regions to assess variation within alfalfa populations, and to develop a measure of relationships among alfalfa accessions from the *M. sativa* core collection.

Materials and methods

A partial *M. sativa* chloroplast genome restriction map and cloned fragments covering nearly the entire genome, provided by L.B. Johnson, Kansas State University, were used in the initial stages of this project. The hypervariable region described by Johnson and Palmer (1989) was revealed in *Hae*II-digested DNA, and was located on a 3.9-kb *Eco*RV fragment. The appropriate fragment was subcloned into pBluescript S K-(Stratagene, La Jolla, Calif.) and partially sequenced by the Iowa State University Biotechnology facility, Ames, Iowa. Similarly, the hypervariable region described by Schabel (1995) was determined to reside on a 0.8-kb *Hin*dIII fragment which was subcloned and completely sequenced. The *Hin*dIII region is known to occur in a 4-kb region between the *psbN* and *rps12* genes, while the *Hae*II region occurs in a 4-kb region between the *trnL* and *rps19* genes, based on observed gene synteny between tobacco (Wakasugi et al. 1998) and alfalfa chloroplast genomes (L. B. Johnson, personal communication). PCR primers were manually designed. Care was taken to match the melting temperatures of the primers for both regions such that one amplification profile could be used for both regions. The primer sequences for the *Hin*dIII region were: forward: (5´CAGAAGAAGCTCAAGCCCAC3´) and reverse: (5´GGCGTAACAAACATTGTTGC3´). The primer sequences for the *Hae*II region were: forward: (5´ATAGTTCAAACCGCACTC3´) and reverse: (5´CGTACCTCCCATAAAGTC3´). The 50-µl PCR reaction mixtures consisted of 0.005 mg of BSA, 20 mM $(NH_4)_2SO_4$, 0.05 M TRIS-HCl (pH 9.0, 25 C), 200 µM each of dATP, dCTP, dGTP and dTTP, 3 mM $MgCl_2$, 25 ng of template DNA and 0.1 U of Tfl polymerase (Epicentre, Madison, Wis.). The amplification profile used was: initial denaturation at 95°C, 3 min, then 49 cycles of 95°C 30 s, 48°C, 1 min, and 72°C, 2 min. PCR fragments of interest were cloned with the TOPO-TA kit (Invitrogen, Carlsbad Calif.). Sequencing was carried out by the Iowa State University Biotechnology facility, Ames, Iowa.

The plant material used in this study consisted of 135 alfalfa accessions from the core collection of the U.S. Department of Agriculture's alfalfa germplasm collection. The region of origin of each accession was assigned based on the reported country of origin, and the associated region (Barnes et al. 1977).

Seeds were scarified with sandpaper and germinated on moist paper towels in closed plastic boxes. DNA was extracted by the method described by Dellaporta et al. (1983) from seedlings with fully expanded cotyledons.

PCR products from the *Hin*dIII region were resolved on 2% agarose (Seakem LE, FMC, Rockland, Me.) with 0.3% Synergel (Diversified Biotech, Boston, Mass.). PCR products from the *Hae*II region were resolved on 1.5% agarose. Size standards included on each gel were Lambda DNA digested with *Bgl*I. Gel images were captured with a digital camera and processed with 1D software (Kodak, Rochester N.Y.), which generated a table of sizes representing each PCR fragment on the gel.

Data were analyzed with the SAS system (1989a, b) using programming steps written by the author. Preliminary investigations indicated that the variation in the size of a fragment, from gel to gel as determined by the 1D software, was less than 50 bp. Therefore, the steps in the analysis consisted of: (1) the reported size of each fragment was rounded to the nearest 50 bp, (2) the proportions of shared fragments among all possible pairs of plants were determined and a distance measure (1 - proportion shared) was generated, (3) a principal component analysis was carried out on the distance data, and (4) a cluster analysis using the agglomerative "Wards" method of PROC CLUSTER was carried out on the distance data. The number of clusters present was judged from a consensus of two clustering statistics, the pseudo F and pseudo t^2. Proceeding from the largest number of clusters to one cluster, a local maximum of pseudo F, concomitant with a local minimum of pseudo t^2, followed by a much larger value of pseudo t^2 with the next cluster fusion, indicated the number of clusters present (SAS, 1989b, page 98).

The diversity within each accession was measured with the Shannon diversity index: $H=-\Sigma[(p_i)(\ln(p_i)]$, where p_i indicates the frequency of each size of cpDNA fragment within the accession. The normality of the distribution of the indices was tested with the Shapiro-Wilk (Wilk 1965) test.

To evaluate the stability of the hypervariable regions through the sexual phase of the plant, the hypervariable segments from a set of parents and 40 progeny were amplified. To evaluate the stability of the regions through vegetative cycles of plant growth, the hypervariable segments were amplified from a set of 20 ramets from each of two genotypes. The ramets had been grown for 4 years (2 years in a greenhouse and 2 years in the field), for a total of 80 plant-years of growth for each genotype.

Results and discussion

Amplification of the *Hae*II region resulted in 43 different size fragments with a frequency weighted average size of about 1300 bp (Table 1; example gel in Fig. 1a). Amplification of the *Hin*dIII region resulted in 23 different fragments with a frequency weighted average size of about 650 bp (Table 1; example gel in Fig. 1b). Evaluation of both hypervariable regions for stability through the sexual phase of the plant indicated complete stability. Forty progeny plants had the hypervariable fragment from one or both parents. Evaluation of the *Hae*II region for stability through cycles of vegetative growth of the plant also indicated complete stability. However, the *Hin*dIII region had changed size in one of the 20 vegetative ramets of one genotype (Fig. 2). Comparison of the DNA sequences of the original and the shorter fragment revealed that the shorter fragment differed from the original by the loss of 145 contiguous base pairs (Fig. 3). The entire hypervariable fragment contained numerous, short repeated elements. For example, the sequence TTTAACTTATTTACTT occurred 16 times in the original fragment, and many more short repeats could be described. The region involved in the deletion was composed of two identical, tandemly arrayed direct repeat elements of 146 bp each (Fig. 3). The deletion was the result of the loss of the DNA between the 53rd base of the first element, and the 52nd base of the second (Fig. 3). The resulting shorter fragment contained essentially one intact 146-bp repeat, instead of two, but with one additional base, the original 53rd base from the first element. This kind of deletion is typical of replication slippage er-

ror, suggesting that the *Hin*dIII region had undergone a replication-related deletion one time within 80 plant-years of growth. The deletion has been observed only in this one plant. The plant carrying the deletion apparently had only the novel size of fragment (Fig. 2), suggesting the plant had become homoplastic for chloroplasts with the shortened fragment. Wolfson et al. (1991) suggested that replication slippage is a common mechanism of chloroplast genome evolution. The original and novel fragments have been entered into Genbank with the accession numbers AF237706 and AF237707, respectively.

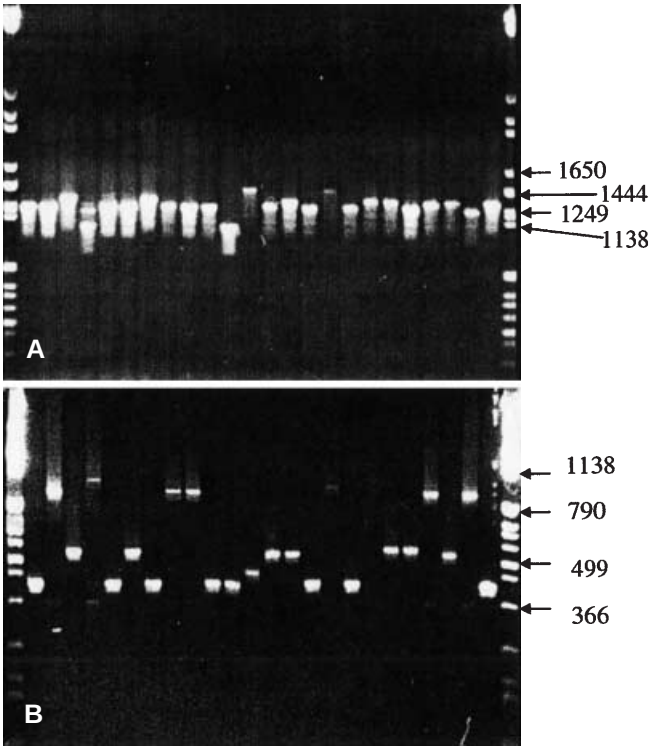


Fig. 1A, B Gel images of PCR products from two hypervariable DNA regions in the alfalfa chloroplast genome. **A** *Hae*II regions from 24 plants of alfalfa accession PI430553 and **B** *Hin*dIII regions from 24 plants of alfalfa accession PI 467916. Fragment sizes are indicated in base pairs

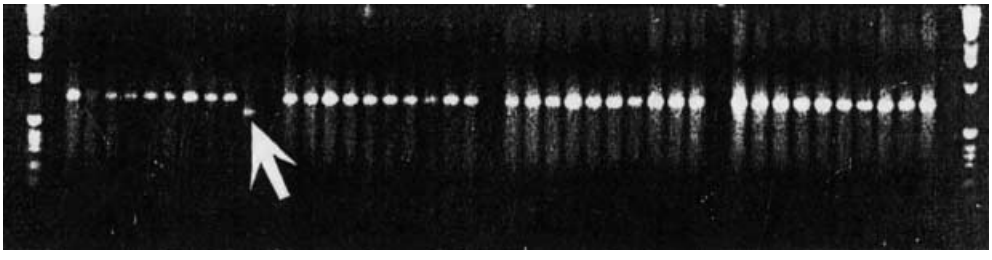
Fig. 2 Gel image of PCR products from the *Hin*dIII hypervariable region of the alfalfa chloroplast genome. Amplification products are from 40 plants comprising two groups of 20 ramets from each of two 'Riley' genotypes. The ramets were grown for 4 years each for a total of 80 years of plant growth for each genotype. The *arrow* indicates the PCR product from one plant that has undergone a deletion in the hypervariable region

The diversity indices from individual accessions ranged from 0.75 to 2.74 with a mean of 1.75. A test for normality indicated that the distribution of diversity indices followed a normal distribution (W=0.97, *P*=0.20). Therefore, it appeared that the accessions included in

Table 1 Sizes and frequencies of PCR fragments at two hypervariable regions of the *M. sativa* chloroplast genome

*Hae*II region[a]		*Hin*dIII region[a]	
Size (bp)	Frequency	Size (bp)	Frequency
600	1	150	1
750	1	200	1
800	1	250	19
850	3	300	32
900	3	350	202
950	1	400	325
1050	34	450	394
1100	49	500	303
1150	235	550	678
1200	608	600	350
1250	743	650	246
1300	691	700	359
1350	442	750	415
1400	174	800	302
1450	60	850	347
1500	38	900	336
1550	24	950	137
1600	16	1000	71
1650	7	1050	28
1700	4	1100	8
1750	1	1150	5
1900	2	1200	4
1950	3	1250	1
2050	1		
2100	1		
2150	2		
2200	1		
2300	3		
2450	1		
2700	1		
2900	1		
3250	1		
3650	1		
3750	1		
3800	2		
3850	2		
3900	3		
3950	3		
4100	2		
4300	1		
4450	1		
4650	2		
5700	1		

[a] Regions were named for the restriction enzyme used in their discovery



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          1                                                          60
Original  GGCGTAACAA ACATTGTTGC CATATAATTT TCCTCATATA ATTTGAGATA AGTTGATTTT
Novel     GGCGTAACAA ACATTGTTGC CATATAATTT TCCTCATATA ATTTGAGATA AGTTGATTTT
          61                                                         120
Original  GTTTCCTTTA TTTATTTTTT TTTACTTTTT TAACTTATTT ACTTTTTTAA CTTATTTATT
Novel     GTTTCCTTTA TTTATTTTTT TTTACTTTTT TAACTTATTT ACTTTTTTAA CTTATTTATT
          121                                                        180
Original  TTTTTTTACT TTTTTAACTT ATTTACTTTT TTAACTTATT TATTTTTATT TACTTTTTTA
Novel     TTTTTTTACT TTTTTAACTT ATTTACTTTT TTAACTTATT TATTTTTATT TACTTTTTTA
          181                                                        240
Original  ACTTATTTAC TTTTTTAACT TATTTACTTT TTTAACTTAT TTACTTTTTT AACTTATTTA
Novel     ACTTATTTAC TTTTTTAACT TATTTACTTT TTTAACTTAT TTACTTTTTT AACTTATTTA
          241                                                        300
Original  CTTTTTTAAC TTATTTACTT TTTTAACTTA TTTACTTTTT TAACTTATTT ATTTTTTTTT
Novel     CTTTTTTAAC TTATTTACTT TTTTAACTTA TTTACTTTTT TAACTTATTT ATTTTTTTTT
          301                                                        360
Original  TACTTTTTTA ACTTATTTAC TTTTATTTAC TTTTTTAACT TATTTACTTT ATTTATTTAC
Novel     T--------- ---------- ---------- ---------- ---------- ----------
          361                                                        420
Original  TTACTTTATT TATTTACTTT ATTTAacttt tttaacttat ttacttttt aacttattta
Novel     ---------- ---------- ---------- ---------- ---------- ----------
          421                                                        480
Original  ctttttttaac ttatttattt tttttttact tttttaactt atttactttt atttactttt
Novel     ---------- ---------- ------TACT TTTTTAACTT ATTTACTTTT ATTTACTTTT
          481                                                        540
Original  ttaacttatt tactttattt atttacttac tttatttatt tactttattt aACTTTTTTA
Novel     TTAACTTATT TACTTTATTT ATTTACTTAC TTTATTTATT TACTTTATTT AACTTTTTTA
          541                                                        600
Original  ACTTATTTAC TTTATTTAAC TTATTCATTT ACTTACTTTA TTTAACTTAT TTATTTACTT
Novel     ACTTATTTAC TTTATTTAAC TTATTCATTT ACTTACTTTA TTTAACTTAT TTATTTACTT
          601                                                        660
Original  ACTTTATTTA ACTTATTTAT TTACTTACTT TATTTATTTA CTTTATTTAA CTTATTCATT
Novel     ACTTTATTTA ACTTATTTAT TTACTTACTT TATTTATTTA CTTTATTTAA CTTATTCATT
          661                                                        720
Original  TACTTACTTT ATTTATTTAC TTTATTTAAC TTATTTACTT ATTTAACTTT GTTTAATCTG
Novel     TACTTACTTT ATTTATTTAC TTTATTTAAC TTATTTACTT ATTTAACTTT GTTTAATCTG
          721                                  759
Original  CGACCGTATC AACAATTCCG TGGGCTTGAG CTTCTTCTG
Novel     CGACCGTATC AACAATTCCG TGGGCTTGAG CTTCTTCTG
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Fig. 3 Aligned cpDNA sequences of original and novel PCR fragments from a hypervariable region of the alfalfa chloroplast genome. The novel fragment arose via apparent deletion from the original form during vegetative growth. Tandem repeats associated with the deletion are *underlined* (first copy) and in *lowercase* (second copy)

this study represented a normally distributed sample of the ranges of intra-accession diversity found within the USDA alfalfa collection.

Genetic distance tables were generated from the fragment data from each of the two hypervariable regions. The correlation of these distance measures was 0.17 (data not shown). Because all possible combinations of the 135 accessions were calculated, 18,225 comparisons were made. With over 18,000 degrees of freedom, an r value of 0.17 is significant at $P<0.05$. However, an r value of 0.17 accounts for less than 3% of the variation in a linear relationship. Therefore, these two measures of distance were considered to be essentially independent, and were treated as two separate measures of the relationship among alfalfa accessions.

Using the genetic distances as variables in a cluster analysis (Ward's minimum variance method) resulted in a dendrogram indicative of geographic differentiation. For example, of the 12 plant accessions from Morocco, five formed an independent group and nine occurred among a group of 12 accessions (Fig. 4). Three other accessions from Morocco were widely separated from the other nine, suggesting that at least two lineages comprise the accessions from Morocco. The clustering statistics formed a local maximum of pseudo F concomitant with a local minimum of pseudo t^2 followed by a much larger value of the pseudo t^2 at the next cluster fusion, only at the transition from two to one cluster (Fig. 5A), indicating that the accessions from Morocco comprised two distinct clusters.

In comparing the positions in a graph of the first three dimensions from the principal component analysis of accessions from Morocco to other accessions from the African region of origin (Fig. 6), it appeared that most Moroccan accessions were quite distinct from the other African accessions. This finding is in agreement with Warburton and Smith (1993) who suggested at least six distinct lineages were present in accessions from the African continent and associated areas.

Of the six accessions from Peru, the origin of another of the nine basic germplasm sources, three occurred within a group of four accessions, while the remaining three were somewhat dispersed in the dendrogram (Fig. 4). There was no clear indication of more than one cluster for Peru (Fig. 5b) suggesting that a single lineage comprised the Peruvian accessions. This result is in agreement with Kidwell et al. (1994) who found their plants of Peruvian origin tended to cluster together, while most plants of other origins did not.

The accessions of Flemish origin (four accessions from France), while not tightly clustered in the dendrogram (Fig. 4), did fall within close proximity to one another in the three-dimensional graph from the principal component analysis (Fig. 7), and the clustering statistics indicated a single cluster (data not shown), indicating that the four accessions were probably of the same lineage.

Of the remaining accessions that could be considered to have originated from the basic germplasms sources, distinct groups were not apparent from the dendrogram. For example, there were 18 accessions from Turkey, part of the Turkistan region of origin (Barnes et al. 1977). From the dendrogram (Fig. 4), no relationship of these 18 accessions was readily apparent. However, on viewing a graph of the first three dimensions from the principal component analysis of the genetic distance data (Fig. 8), it appeared that nearly all of the accessions from Turkey formed two relatively large groups, with a few accessions quite distant from the main body of accessions from Turkey, and from each other (Fig. 8) . The clustering statistics for Turkish accessions (Fig. 5c) indicated that either five or three clusters were present. Considering the evidence from the three-dimensional graph (Fig. 8), and the clustering statistics (Fig 5c), there appeared to be evidence of five distinct lineages within the

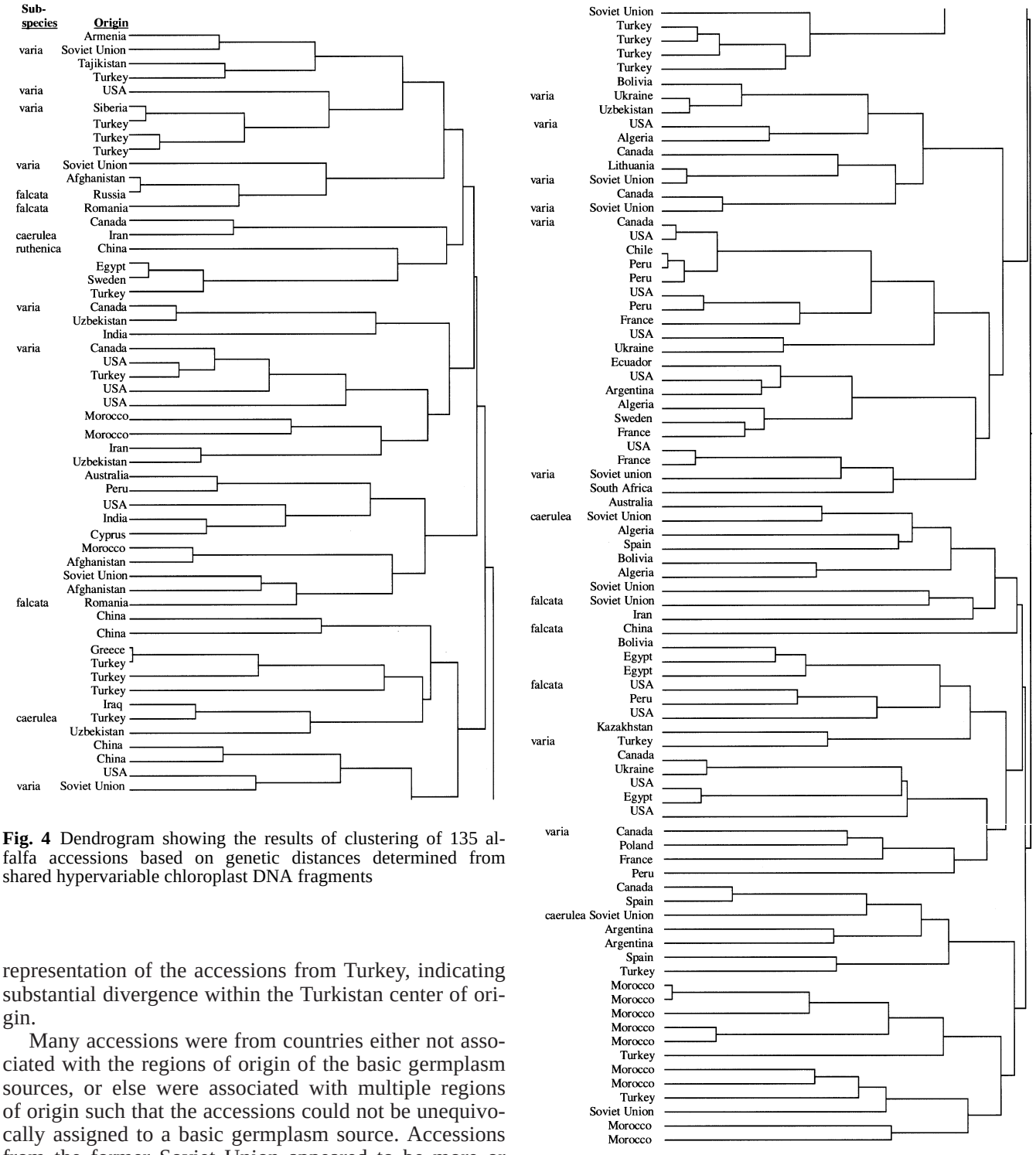

Fig. 4 Dendrogram showing the results of clustering of 135 alfalfa accessions based on genetic distances determined from shared hypervariable chloroplast DNA fragments

representation of the accessions from Turkey, indicating substantial divergence within the Turkistan center of origin.

Many accessions were from countries either not associated with the regions of origin of the basic germplasm sources, or else were associated with multiple regions of origin such that the accessions could not be unequivocally assigned to a basic germplasm source. Accessions from the former Soviet Union appeared to be more or less randomly distributed throughout the dendrogram (Fig. 4). A graph of the first three principal components of genetic distance data failed to indicate any distinct groups within the Soviet accessions (Fig. 9). The clustering statistics for the Soviet accessions (Fig. 5d) suggested there may be two groups present. These results were interpreted to indicate that the accessions from the Soviet Union represented a heterogeneous mixture of genotypes, possibly representing two broad groups, but with no clear indication of distinctive lineages. The regions of origin of the *Medicago falcata* and *Medicago varia* sources overlap over a large portion of the former Soviet Union (Barnes et al. 1977). Hence, it is possible that the groups indicated by the clustering statistics may reflect this heterogeneity.

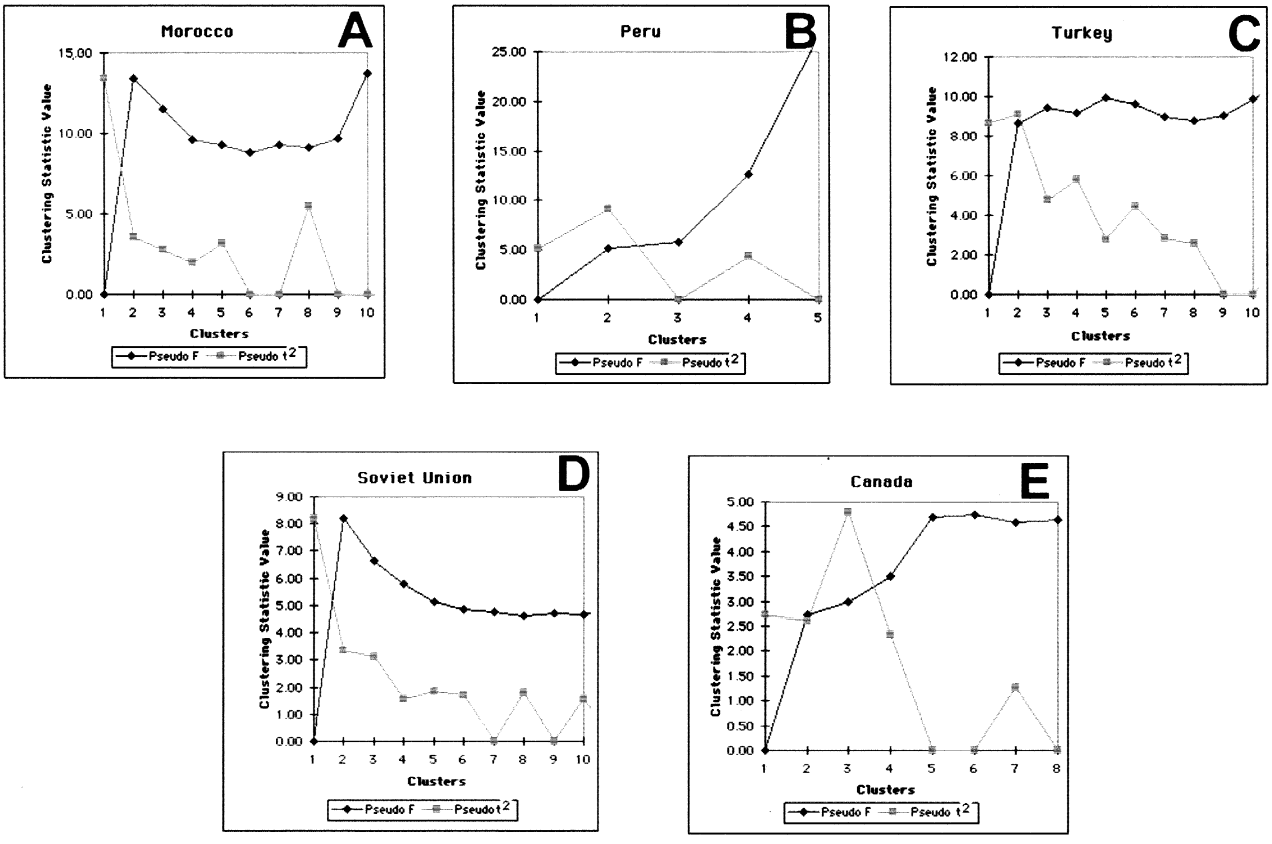

Fig. 5A–E Clustering statistics for alfalfa accessions from the indicated countries, based on genetic distances determined from shared hypervariable chloroplast DNA fragments

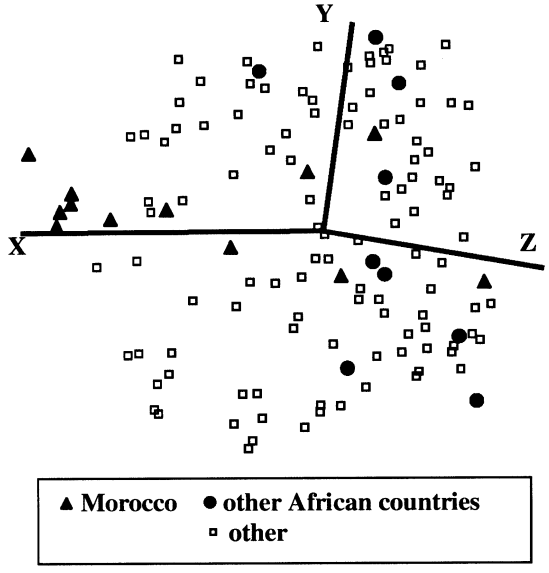

Fig. 6 Plot of the first three dimensions from a principal component analysis of genetic distances among alfalfa accessions from Morocco and other African nations, determined from shared hypervariable chloroplast DNA fragments

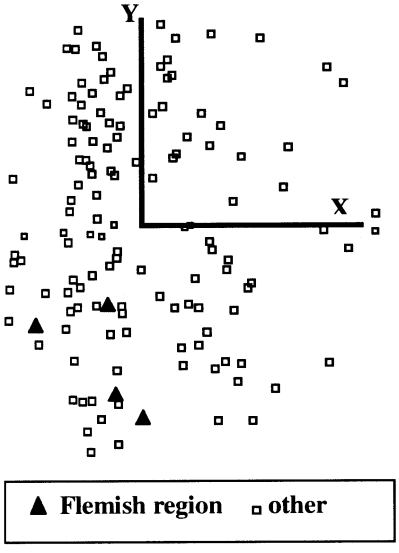

Fig. 7 Plot of the first three dimensions from a principal component analysis of genetic distances among alfalfa accessions from countries associated with the Flemish region of origin, determined from shared hypervariable chloroplast DNA fragments

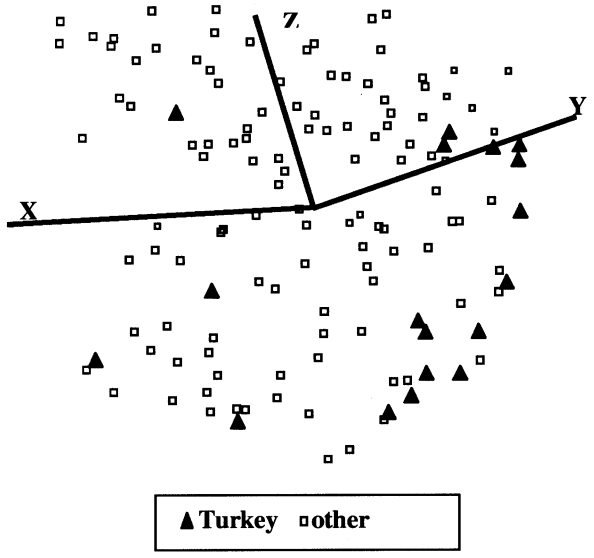

Fig. 8 Plot of the first three dimensions from a principal component analysis of genetic distances among alfalfa accessions from Turkey, determined from shared hypervariable chloroplast DNA fragments

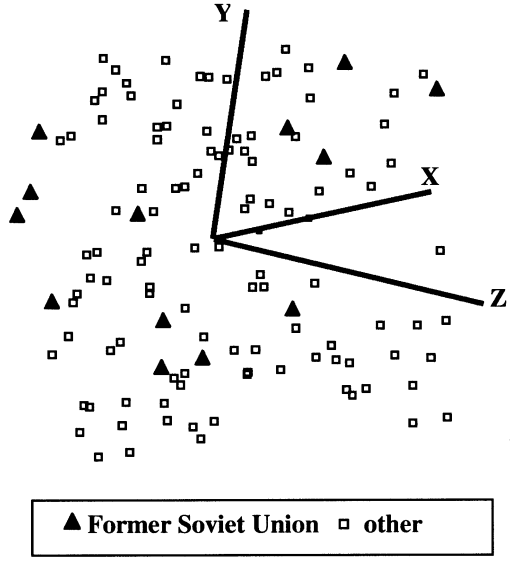

Fig. 9 Plot of the first three dimensions from a principal component analysis of genetic distances among alfalfa accessions from countries formerly part of the Soviet Union, determined from shared hypervariable chloroplast DNA fragments

In contrast to the result of the accessions from the former Soviet Union, the clustering statistics for the accessions from Canada clearly indicated the presence of five clusters (Fig. 5e). This result demonstrated that the accessions from Canada were quite distinct from one another, indicating that the accessions collected from that country may have represented multiple introductions, or else substantial divergence due to selection pressures. A similar result was seen with the 29 accessions from the United States. The clustering statistics for the U.S. accessions indicated that at least three distinct lineages were present (data not shown). Taken together with the results from the Canadian accessions, this result suggests that the accessions collected in North America and included in the core collection represented at least eight distinct lineages.

Of six accessions from China, four were relatively closely related, occurring within 11 accessions of one another in the dendrogram (Fig. 4). The other two were quite distinct from the first four accessions from China and from each other (Fig. 4). The two unusual accessions, PI 491407and PI 502645, were classified as *M. falcata* and *Medicago ruthenica*, respectively. The other four accessions from China were classified as *M. sativa*; hence, the distinction of these six accessions into three groups was probably due to subspecies differentiation.

In general, however, the accessions that were classified as a subspecies other than *M. sativa* did not cluster in any readily apparent fashion, with the possible exception of small groups of *M. falcata* accessions (Fig. 4). This result suggested that the polymorphisms in the hypervariable chloroplast DNA regions occurred with frequencies essentially independent of the subspecies classification, and continued to diverge after the evolution of the subspecies. The indication of instability of at least one of these hypervariable fragments through the vegetative growth phase of the plant (Fig. 2) is consistent with this possibility.

Based on these results it is suggested that fragment length polymorphisms in hypervariable regions of the alfalfa chloroplast are useful for distinguishing populations. The results also suggest that substantial genetic diversification has occurred within some of the basic germplasm sources, such as the Turkistan region. At least some of this diversification may result from physical alteration of the hypervariable cpDNA regions during vegetative growth. Results from other regions, such as the Flemish and Peruvian regions, appeared to be consistent with the presence of a single lineage. It also appears that alfalfa accessions collected from regions not associated with the basic germplasm origins, such as North America, tend to be quite divergent, probably as a result of multiple introductions and diversification due to local environments.

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