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RFLP variation in diploid and tetraploid alfalfa

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Summary. Alfalfa (*Medicago sativa* L.) is a major forage crop throughout the world. Although alfalfa has many desirable traits, continued breeding is required to incorporate pest resistances and other traits. We conducted this study to determine the amount of restriction fragment length polymorphism (RFLP) variability present within and between diploid and tetraploid alfalfa populations, and whether or not this variability is sufficient for construction of an RFLP map. Diploid plants from *M. sativa* ssp. *falcata*, ssp. *coerulea*, and ssp. *sativa* and tetraploid spp. *sativa* cultivars 'Apollo,' 'Florida 77,' and 'Spredor 2' were included. A total of 19 cDNA clones was probed onto genomic Southern blots containing DNA digested by *Eco*RI, *Hin*dIII, or *Bam*HI. Phylogenetic trees were produced, based on parsimony analysis of shared restriction fragments. Evidence for extensive gene duplication was found; most probes detected complex patterns of restriction fragments. Large amounts of variation are present within all diploid subspecies. *M. sativa* ssp. *falcata* plants formed clusters distinct from ssp. *sativa* or ssp. *coerulea* plants, which were not distinctly clustered. Some *M. sativa* ssp. *falcata* plants were more similar to the other groups than to other plants within ssp. *falcata*. Variation among tetraploid cultivars showed that Florida 77 and Apollo had more similarities than either showed with Spredor 2. All three cultivars showed large within-population variation, with Apollo being the most diverse and Spredor 2 the least. Based on these results, development of an RFLP map at the diploid level appears possible. Also, differentiation of cultivars, particularly ones of divergent origin, seems possible based on RFLP patterns.

Key words: RFLP – Alfalfa – Genetic diversity – Phylogenetic tree – Gene duplication

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Introduction

Alfalfa (*Medicago sativa* L.) originated in the Caucasus, northeastern Turkey, and northwestern Iran and over 32 million hectares are currently cultivated on all continents of the world (Michaud et al. 1988). Among the important characteristics of alfalfa are its high forage quality (protein, vitamins, and minerals), its ability to fix atmospheric N_2, its positive effect on soil tilth, and its utility as a model system for genetic studies of autotetraploid species. However, continued alfalfa breeding is necessary to develop pest resistances, overcome bloat, increase N_2 fixation, and maximize yield (Barnes et al. 1988).

Medicago sativa consists of a complex of several subspecies, both diploids and tetraploids, which are all interfertile and have the same karyotype (Quiros and Bauchan 1988). Some *M. sativa* ssp. *falcata* accessions and ssp. *coerulea* are diploids, but other *M. sativa* ssp. *falcata* accessions, as well as ssp. *sativa* and ssp. *glomerata*, are tetraploids. Cultivated alfalfa (*M. sativa* ssp. *sativa*) is an autotetraploid (Stanford 1951). Bingham and McCoy (1979) developed Cultivated Alfalfa at the Diploid Level (CADL) by mating haploids from cultivated alfalfa with *M. falcata* and repeatedly backcrossing to, or intermating with, haploids derived from cultivated germplasm. This population is reproductively stable at the diploid level, enabling *M. sativa* ssp. *sativa* to be studied at two ploidy levels. Another diploid population, W2xiso, was derived from eight CADL plants by making four single crosses, two double crosses, and one double double cross (Bingham 1991).

Cultivated alfalfa is cross-pollinated by bees and cannot withstand much inbreeding (Rumbaugh et al. 1988). Although self-fertility varies among plants, development of inbred lines is difficult. Therefore, breeding methods used to develop cultivars usually focus on intermating

various plants (e.g., strain crosses or synthetics), rather than creating inbred lines which could be used for hybrid production.

Molecular genetic characterization of alfalfa has lagged behind other major crops. Currently, no genetic map, or even linkage groups, have been defined. An RFLP map, such as those constructed for other crops [e.g., rice (McCouch et al. 1988), maize (Heletjaris et al. 1986), and tomato (Bernatzky and Tanksley 1986b)], would be useful for a wide range of breeding applications. First, an RFLP map could facilitate introgression of desired genes from less agronomically useful alfalfa taxa. For example, a major problem of alfalfa production, particularly in the southern USA, is the alfalfa weevil (*Hypera postica* Gyllenhal). Sources of resistance are not present in commercially available alfalfa cultivars but have been found in some related taxa such as *M. rugosa, M. scutellata,* and *M. glandulosa* (Sorenson et al. 1988). Linking genes controlling resistance to RFLP markers would allow efficient scoring for desired introgressed chromosomal fragments while eliminating undesired fragments. Second, a map may help breeders choose the best potential parents for the development of new cultivars, possibly as an aid to maximize heterozygosity (Dunbier and Bingham 1975).

Outcrossing plants have been shown to be highly polymorphic, even between accessions within subspecies of *Brassica* (Figdore et al. 1988) and among maize (*Zea mays* L.) yellow dent cultivars (Shattuck-Eidens et al. 1990). In contrast, inbreeding crops such as peanut [*Arachis hypogaea* L. (Kochert et al. 1990)], melon [*Cucumis melo* (Shattuck-Eidens et al. 1990)], and soybean [*Glycine* subg. *soja* (Keim et al. 1989)] generally exhibit little RFLP variation. This study was conducted to determine if large amounts of variability are present within and among various alfalfa populations at both the diploid and tetraploid levels, as would be expected based on its breeding system. The long-term goal of our breeding program is to develop an RFLP map that can be applied to a number of cultivar improvement objectives. We conducted this experiment to determine if sufficient variability exists among diploid populations to enable mapping. Additionally, we attempted to quantify the level of heterozygosity present among tetraploid and diploid populations by comparing the number of bands produced by various probe/enzyme combinations.

Materials and methods

Plant materials used in this study are shown in Table 1. Diploid plants were chosen to represent germplasm from three subspecies within the *M. sativa* complex: six plants of ssp. *falcata*, seven plants of ssp. *coerulea*, 23 plants of ssp. *sativa* [CADL (20) and W2xiso (3)], and four plants representing crosses between ssp. *falcata* and ssp. *sativa*. A total of 20 tetraploid plants was randomly selected from each of the cultivars Apollo, Florida 77, and Spredor 2 growing in field plots at the University of Georgia Plant Science Farm near Athens/GA. The three cultivars were chosen because of their diverse genetic backgrounds.

Table 1. Plant material used in RFLP studies

No. plants	Subspecies	PI/Name
1	*falcata*-2x	PI251830
1	*falcata*-2x	PI262532
1	*falcata*-2x	PI314093
1	*falcata*-2x	PI315456
1	*falcata*-2x	PI464727
1	*falcata*-2x	PI464729
1	*falcata* × *sativa*-2x[a]	PI307098
1	*falcata* × *sativa*-2x	PI440540
1	*falcata* × *sativa*-2x	PI468014
1	*falcata* × *sativa*-2x	PI346923
2	*coerulea*-2x	PI179370 nos. 1 & 2
1	*coerulea*-2x	PI440501 no. 2
1	*coerulea*-2x	PI440512 no. 2
1	*coerulea*-2x	PI464718 no. 1
1	*coerulea*-2x	PI464710 no. 1
1	*coerulea*-2x	PI464724 no. 1
20	*sativa*-2x	CADL[b]
3	*sativa*-2x	W2xiso[c]
20	*sativa*-4x	Apollo
20	*sativa*-4x	Florida 77
20	*sativa*-4x	Spredor 2

[a] Seed for the *M. sativa* ssp. *falcata* × *sativa* plants was labelled as the indicated PI, but the plants produced purple flowers, indicating that some outcrossing, presumably with ssp. *sativa*, had occurred during seed production

[b] Cultivated alfalfa at the diploid level (Bingham and McCoy 1979)

[c] Bingham 1991

Genomic DNA was extracted from freeze-dried leaves of a single plant by the CTAB technique of Saghai-Maroof et al. (1984). Agarose electrophoresis, hybridization, and RFLP analysis generally followed the protocols of McCouch et al. (1988). Genomic DNA was digested with one of three different enzymes (*Eco*RI, *Hin*dIII, or *Bam*HI), separated by electrophoresis in 0.8% agarose gels (10 µg/gel lane), and blotted onto GeneScreen Plus (DuPont) or Immobilon-N (Millipore) nylon membranes by the method of Southern (1975). A total of 19 randomly chosen clones (0.5–2 kb) was selected from two cDNA libraries in λ Zap II (obtained courtesy of Dr. G. Bauchan, USDA-ARS, Beltsville/MD). Libraries were constructed from alfalfa seedlings which were either challenged with anthracnose (*Colletotrichum trifolii* Bain.) or unchallenged. Probes were labeled I or U for induced or uninduced library, respectively. Each of the clones was screened with cotton chloroplast DNA, and U039 was found to be a chloroplast probe. Cloned inserts were amplified from the λ ZAP II vector by the polymerase chain reaction using T3 and T7 17-mers, hexamer-labelled with ^{32}P-dCTP to high specific activity (Feinberg and Vogelstein 1984), and hybridized at 65 °C overnight to the genomic DNA nylon membranes. Membranes were washed at 65 °C with 2 × SSC + 0.1% SDS (once) and 1 × SSC + 0.1% SDS (twice) for 20 min each. Membranes were subsequently wrapped in Saran Wrap and exposed to Kodak X-OMat film using one intensifying screen at -80 °C for 3–7 days.

To facilitate consistent scoring, a common plant was present on all membranes of a given ploidy group. Common fragments

(bands) in different individuals were assumed to represent the same restriction fragment. All fragments scored were considered 'unit characters' since no inference about allelism could be made. Data were organized into two 1-0 matrices (one for diploids and another for tetraploids), which were analyzed independently with the computer program 'Phylogenetic Analysis Using Parsimony' (PAUP, version 2.4) developed by D.L. Swofford (Illinois Natural History Survey, Champaign/IL). The heuristic method using addition = step-wise and mulpar functions was used to generate phylogenetic trees and pair-wise patristic distance comparisons among plants.

Results and discussion

Level of RFLP variation

An autoradiogram representing 21 diploid plants is shown in Fig. 1. Multiple bands of approximately equal intensity are present in some lanes. These could represent different alleles of the same locus, and thus indicate heterozygosity, or they could be different loci and represent gene duplication. In the absence of segregation analysis, it is difficult to distinguish between these two possibilities. However, in some cases alleles appear to be present in different dosages, as shown by some bands that are darker than others of the same size in different plants, but assignment of alleles is not possible without segregation analysis. Other probes give much more complex patterns on both diploids and tetraploids. For example, probe I013 (Fig. 2a, b) and others show multiple bands and large amounts of apparent heterozygosity in both ploidy groups, as would be expected in an outcrossing species. Variations in intensity of different bands may reflect either allele copy number or degree of homology of various loci to the probe. Many probe/enzyme combinations (P/E) detect bands that are present at a low frequency in the tetraploid population. If the probe can bind weakly to a partially homologous locus, perhaps these alleles can only be detected if they are in the quadriplex state. Quadriplex genotypes are expected to be present at a low frequency (Dunbier and Bingham 1975), so this could account for some of the rare bands. Conversely, rare bands could simply represent a rare allele of a given locus. It is possible that higher washing stringencies would resolve some of these questions since bands resulting from low homology would not be present. We will have to perform segregation analyses to definitively prove the allelic relationships present in these banding patterns.

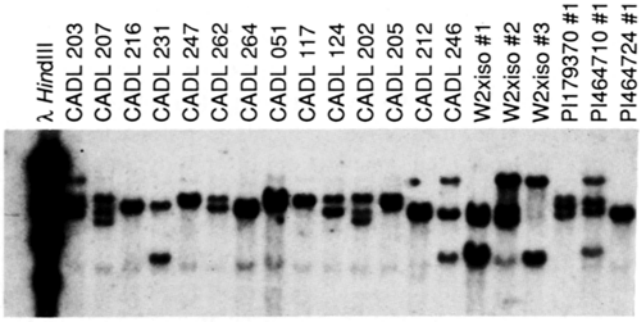

Fig. 1. Autoradiogram derived from probing *Hin*dIII-digested DNA from 21 diploid alfalfa plants with U031, showing a relatively simple banding pattern, indicating possibility of different frequencies of alleles demonstrated by dark and light bands

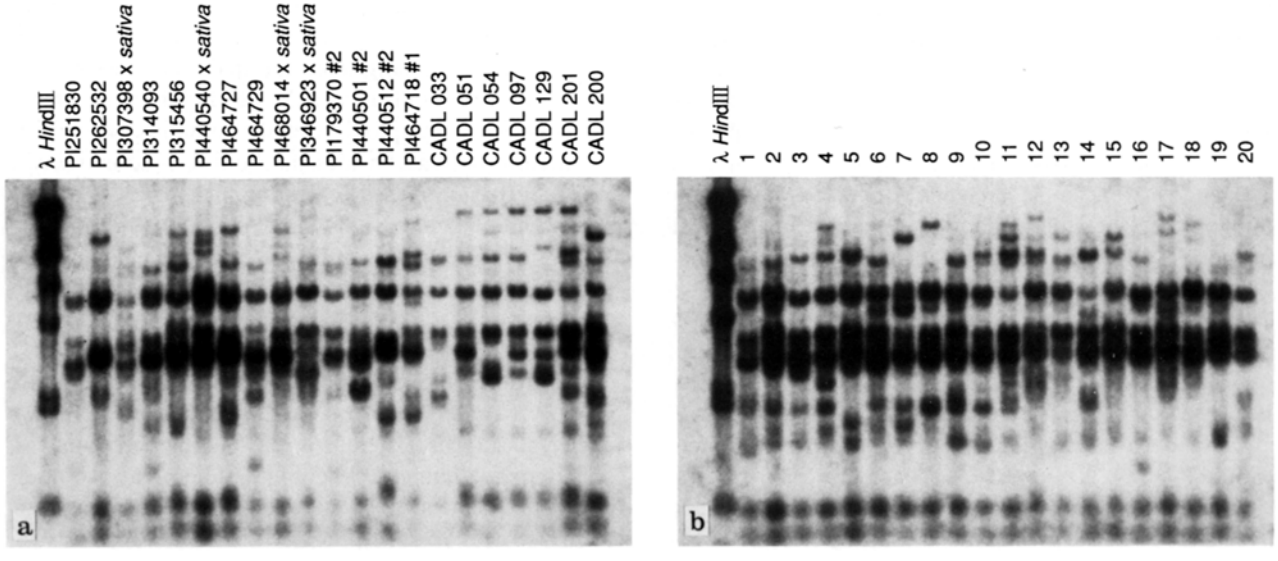

Fig. 2a and b. Autoradiogram derived from probing *Hin*dIII-digested DNA from 21 diploid alfalfa plants (**a**) or 20 Apollo tetraploid alfalfa plants (**b**) with I013, showing a complex patterns of bands of dark and light intensity within a single lane

*Eco*RI produced the most fragments per P/E in both diploids and tetraploids of the three enzymes tested. *Bam*HI produced the fewest and largest fragments (about 10 kb on average). Since scoring large fragments is difficult due to poor resolution, *Bam*HI may not be as useful for alfalfa RFLP studies as *Eco*RI or *Hin*dIII.

The average number of bands per plant for some P/E varied among subspecies and among cultivars. Variation seemed to be random since no group consistently had more or fewer fragments than another. Data for the diploids may be skewed due to the small populations used in this study. Some P/E produce bands that are present in only one subspecies. For example, I009-*Bam*HI produced two fragments unique to CADL, two unique to *M. sativa* ssp. *coerulea* and one unique to ssp. *falcata*. Likewise, some bands are cultivar specific. However, no fragments unique to a particular group are present in all plants of that group, making fingerprinting of cultivars or subspecies [as has been done in rice using a human minisatellite probe (Dallas 1988)] unlikely. Alternatively, it may be possible to differentiate cultivars on a population basis by examining percentages of plants carrying certain fragments. Furthermore, fingerprinting individual plants may be possible with several probes that produced highly complex and unique banding patterns for each plant.

A total of 47 P/E was scorable on the CADL plants studied and 4 (8.5%) were monomorphic; 44 P/E were scorable among tetraploids, with 8 (18.2%) monomorphic. The total number of distinct fragments detected by a P/E within a population was as high as 21 among CADL and 23 among tetraploids. The average number of bands detected per plant ranged from 1 to 8.8 per P/E for CADL and from 1 to 9.2 per P/E for tetraploids. A total of 32 (68.1%) P/E produced an average of two or more bands per CADL plant and 11 (23.4%) produced four or more bands. Among tetraploids, 16 (36.4%) P/E detected four or more bands. A given locus would not be expected to produce more than two bands in a diploid (unless a restriction site was present within the probed fragment) or four bands in a tetraploid. Therefore, the data shown above most likely indicate that many loci are duplicated in the genome. However, segregation data will be necessary to conclusively identify duplicated loci. Quiros and Morgan (1981) reported multiple loci controlling isozymes in two diploid *Medicago* species. McGrath et al. (1990) also found a high level (40%) of duplicated loci detected by cDNA probes in *Brassica*. Bernatzky and Tanksley (1986a) reported that 47% of cDNA clones corresponded to two or more loci in tomato.

Since CADL was developed from tetraploid cultivars, it would not be surprising if the total number of bands seen in the populations of both CADL and the tetraploid cultivars was similar. The CADL plants produced 333 total bands and the tetraploids 427. This result could be biased because of the smaller population size of the CADL plants we sampled. Since only 23 CADL plants were represented compared with 60 tetraploids (each with twice the number of genome equivalents of a diploid), alleles at low frequencies in the population might not have been present. All 40 diploid plants (including *M. sativa* ssp. *falcata* and *coerulea*) studied produced 435 total fragments. Including these subspecies for comparison to the tetraploids may indicate that a larger CADL sample size would eliminate the differences seen. However, the cultivars from which CADL is derived are different from those tested in the present study and this may also account for the lower number of total fragments in CADL.

Maximum heterozygosity (four different alleles at a locus) has been proposed as a method to maximize performance of alfalfa populations (Dunbier and Bingham 1975). A maximally heterozygous tetraploid plant would have four distinct alleles at a given locus whereas a diploid would only have two. Although tetraploids could have up to twice the number of bands as diploids (assuming a P/E detects the same loci in both ploidy groups), it seems unlikely that all loci would be maximally heterozygous. Thus, tetraploids will most likely have less than twice the number of bands per plant present in the diploids. Our data are consistent with this hypothesis, since the tetraploid cultivars produced an average of 3.73 bands per plant compared to CADL, which produced 2.92. Segregation data will allow further clarification of heterozygosity within alfalfa populations. Quiros (1983) reported that tetraploid populations had a higher percentage heterozygosity at two isozyme loci than diploid populations, a result attributed to the higher chromosome number in tetraploids.

It is possible that the actual difference between tetraploids and diploids may be larger than that reported due to artifacts arising from the inability to score faint bands on tetraploid as well as on diploid films. DNA extractions of tetraploids usually contained more contaminating polysaccharides than did diploid extracts, a problem that decreased resolution. This is the case with I009-*Hin*dIII and -*Bam*HI, and possibly the others as well. Alternatively, this difference may indicate the presence of many lethal alleles, which must be masked by other alleles if the plant is to survive. Diploid plants would be expected to maintain two alleles at many loci to screen against the lethals. Tetraploid plants would have no survival advantage in maintaining more than two alleles at a locus. Therefore, the number of fragments seen in individual tetraploid and diploid plants would be closer than expected from random selection, in agreement with our data.

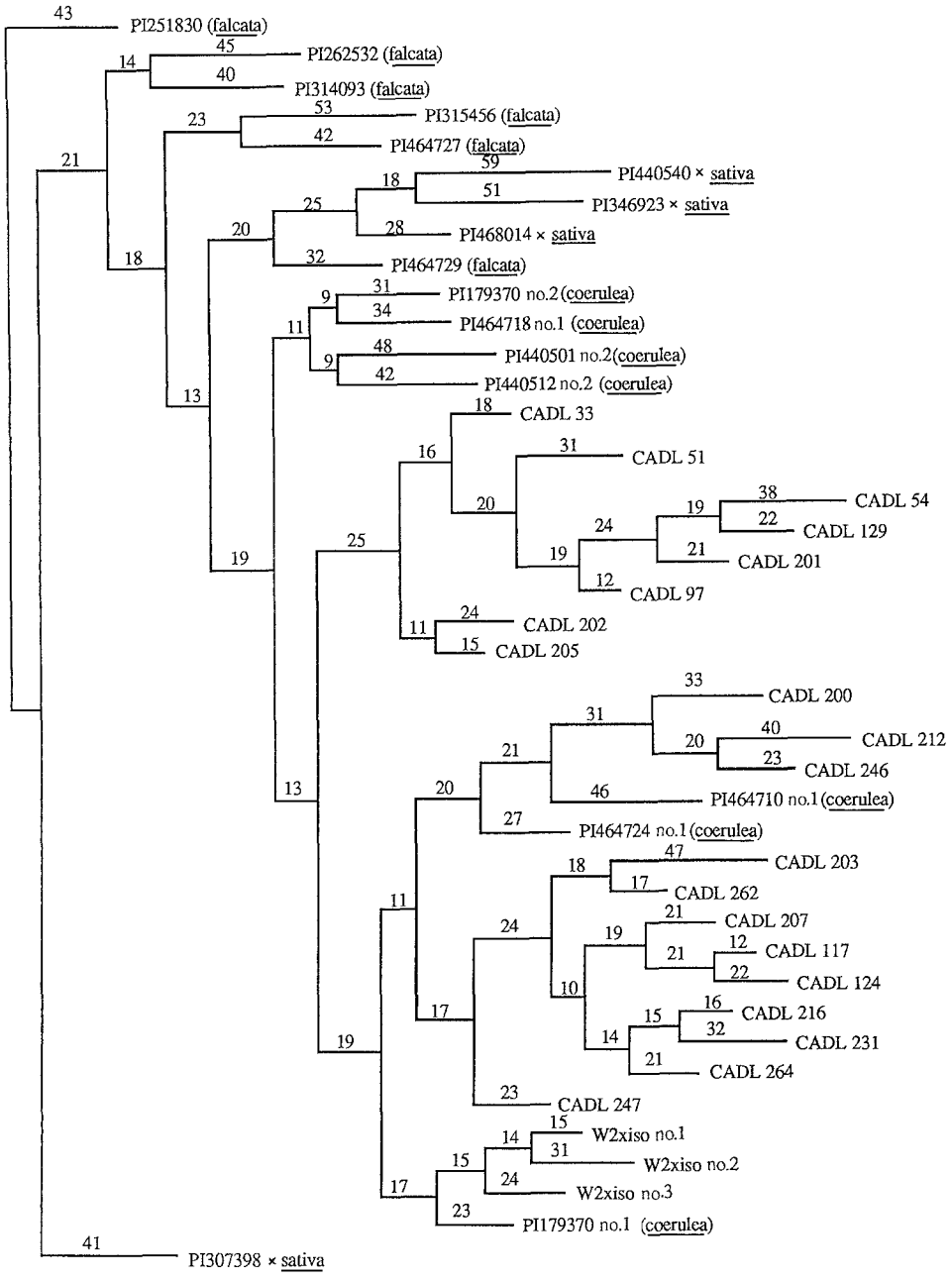

Fig. 3. Unrooted phylogram of 40 diploid alfalfa plants from *M. sativa* ssp. *falcata*, spp. *coerulea*, and ssp. *sativa* (CADL) produced by PAUP based on RFLP data. Tree length = 1,903, consistency index = 0.213. Numbers equal 'unit distances'. Branch length is proportional to the unit distance

Phylogenetic relationships among diploids

The PAUP program constructed only one most parsimonious, unrooted tree from the 435 unit characters scored on diploids (Fig. 3). Distances present on the trees indicate 'unit distances' since the causes of the RFLP differences we detect are unknown (Song et al. 1990). This tree places all *M. sativa* ssp. *falcata* or *falcata* × *sativa* plants on distinct branches from the CADL (ssp. *sativa*) and ssp. *coerulea* plants, the latter two arising from a single node. Subspecies *coerulea* and CADL do not form separate clusters. This is not surprising since the former is the diploid form of purple-flowered *M. sativa* ssp. *sativa* (Quiros and Bauchan 1988) and the latter diploidized from other tetraploid cultivars. Considerable variation occurs both within and between subspecies. Patristic distances within CADL between two plants represented on the tree vary from 34 (CADL 117–CADL 124) to 323

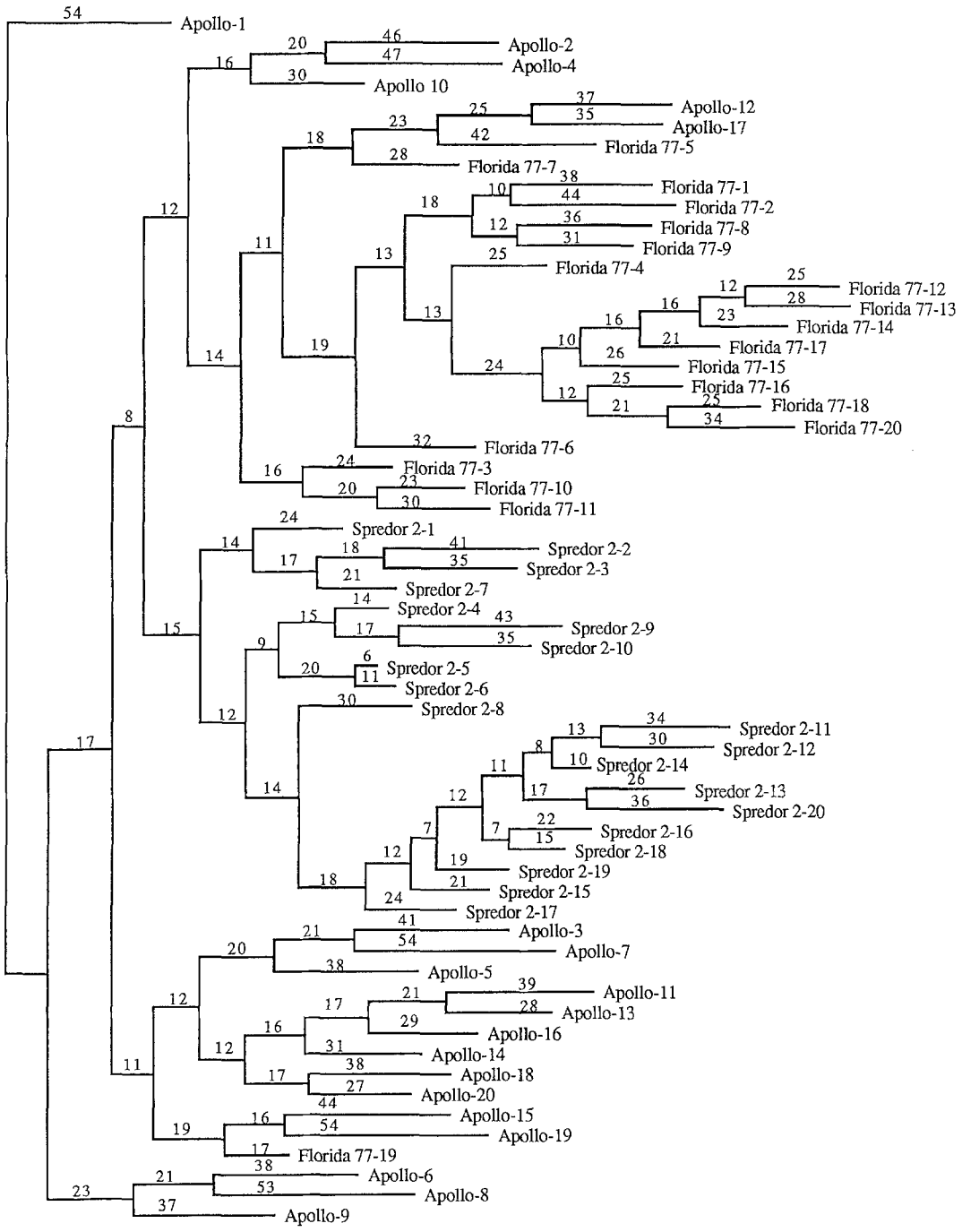

Fig. 4. Unrooted phylogram of 20 tetraploid alfalfa plants from each of three cultivars produced by PAUP based on RFLP data. Tree length = 2,752, consistency index = 0.137. Numbers equal 'unit distances'. Branch length is proportional to the unit distance

(CADL 54–CADL 212). Variation within *M. sativa* ssp. *falcata* and *falcata* × *sativa* plants ranges from 84 (PI251830–PI307398 × *sativa*) to 217 (PI251830–PI440540 × *sativa*). Additionally, some *M. sativa* ssp. *falcata* plants have smaller genetic distances to CADL or *coerulea* than to other *falcata* plants. For example, unit distances for PI464729 (*falcata*) with PI251830 (*falcata*) = 147; with PI179370 no. 2 (*coerulea*) = 122; and with CADL 205 = 135. Therefore, even though much variability exists within the *M. sativa* complex, many similarities are present among subspecies, at least for the accessions tested.

Development of an RFLP map seems feasible given the variation present. Various combinations of these plants have been crossed to produce F_1s which we have selfed to produce an F_2 mapping population. Crosses

exhibit varying levels of self-fertility, but some F_1s are highly self-fertile and able to produce mapping populations of adequate size.

Phylogenetic relationships among tetraploids

The three cultivars studied are all synthetics with different characteristics. Apollo, derived from northern and Flemish-type cultivars, was selected in Iowa for multiple pest resistance and forage yield, and is moderately fall dormant (Moutray 1983). Florida 77 was selected from 100 varieties and introductions selected in Florida for yield and persistence, and is non-fall dormant (Horner 1970; Horner and Ruelke 1981). Spredor 2 was selected for grazing persistence in the northern USA from a number of cultivars and creeping rooted populations, is very fall dormant, and has limited pest resistance (Anonymous 1980). Morphological traits have also been shown to vary among these three cultivars (Brummer and Bouton 1991).

PAUP produced a single, unrooted tree from the 427 unit characters scored on the tetraploid cultivars (Fig. 4). Spredor II plants all branched from a single node. Eighteen Apollo plants clustered, but the remaining two mixed with Florida 77. One Florida 77 plant (19) was in the Apollo cluster. Overall, the cultivars were sufficiently different to form individual clusters. Further P/E combinations may resolve the cultivars into more distinct clusters. Variation present within Apollo is larger than either Florida 77 or Spredor 2 (Table 2). The latter cultivar has the smallest distances between plants. Florida 77 and Apollo have plants more similar to each other (Apollo-15 to Florida 77-19=77) than either cultivar has with Spredor 2. These data show that distinguishing among cultivars, particularly ones of diverse origin, is possible based on RFLP.

Considering the diverse background of the cultivars, it is not surprising that RFLP variability is also present. However, very morphologically diverse accessions of peanut are not variable for RFLPs (Kochert et al. 1990).

Table 2. Maximum and minimum unit distances between plants within and between alfalfa cultivars, as determined by PAUP using patristic pair-wise comparisons

Cultivar comparison	Unit distance	
	Minimum	Maximum
Apollo-Apollo	65	276
Florida 77-Florida 77	53	247
Spredor 2-Spredor 2	17	231
Apollo-Florida 77	77	324
Apollo-Spredor 2	111	296
Florida 77-Spredor 2	108	344

It is likely that mating system plays a large role in determining RFLP variation, with outcrossing plants maintaining large numbers of alleles (e.g., Figdore et al. 1988; Shattuck-Eidens et al. 1990), but alleles in selfing plants [e.g., peanut and soybean (Keim et al. 1989)] would be driven to fixation, thus decreasing polymorphism. Dunbier and Bingham (1975) proposed selection for maximum heterozygosity (four alleles per locus) to increase yield. Using RFLP to tag the genes of interest may enable breeders to realize this goal.

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