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Population structure and combining ability of diverse *Medicago sativa* germplasms

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Abstract Although unadapted germplasms have been used to improve disease and insect resistance in alfalfa, there has been little effort to use these for improving forage yield. We evaluated genetic diversity and combining ability among two unadapted germplasms (*Medicago sativa* ssp. *sativa* Peruvian and *M. sativa* ssp. *falcata* WISFAL) and three Northern U.S. adapted alfalfa cultivars. Population structure analyses indicated that the WISFAL and Peruvian germplasms were genetically distinct from the cultivars, although Peruvian was relatively closer to the cultivars. Peruvian and WISFAL germplasms were intermated to generate a novel hybrid population. This population was crossed to the three cultivars as testers, and the testcross progenies were evaluated for forage yield along with the hybrid population, the original germplasms (Peruvian, WISFAL and cultivars), testcrosses of Peruvian and WISFAL to the three cultivars and a three-way hybrid of the cultivars. The experiment was carried out in the field in Temuco, Chile and Arlington, Wisconsin, USA, and forage was harvested during two seasons. Results from these evaluations showed that hybrids between the Peruvian × WISFAL population and the cultivar testers yielded as much as the cultivar testers. Heterosis was observed between Peruvian and WISFAL, and between these germplasms and the cultivar testers, suggesting that each germplasm may contain different favorable alleles. If Peruvian and WISFAL populations contain alleles at different loci that complement cultivar testers, then combining and enriching these alleles in a single population could result in improved combining ability with alfalfa cultivars.

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Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important legume crops and a superior source of forage due to its high nutritional quality and yield (Sumberg et al. 1983). Although alfalfa has been improved with respect to forage quality (Demment et al. 1986; Sumberg et al. 1983) and pest resistance (Hill et al. 1988), little progress has been made in forage yield. Comparisons between modern and older cultivars have revealed average genetic gains for forage yield of 0.16% per year (Hill and Kalton 1976; Holland and Bingham 1994). This low rate of gain contrasts with higher rates estimated for other biomass crops, such as white clover (0.6%; Woodfield and Caradus 1994) and rye grass (0.5%; van Wijk and Rehuel 1991). Differences are even greater when alfalfa is compared with grain crops such as corn (1.42–1.78%; Duvick 1984) and sorghum (1.3%; Miller and Kebede 1984).

Cultivated alfalfa is an allogamous tetraploid with polysomic inheritance, and individuals can have up to four different alleles at a given locus (Quiros 1982; Stanford 1951). The higher forage yields associated with increased heterozygosity were initially explained by intralocus allelic interaction (overdominance, Bingham 1980). However, observations of severe inbreeding depression (Busbice and Wilsie 1966; Jones and Bingham 1995; Pfeiffer and Bingham 1983), progressive heterosis (Dunbier and Bingham 1975; Groose et al. 1989) and differences in combining ability found between isogenic diploid and tetraploid lines (Groose et al. 1988; Kidwell et al. 1994b) support a model involving complementary gene action in chromosome blocks (Bingham et al. 1994). In this model, the highest yielding genotypes are obtained by combining favorable alleles in repulsion linkage phase within chromosome blocks. Additivity has been shown to be an

important component of genetic variation for forage yield (Kimbeng and Bingham 1998a, b, 1999; Pfeiffer and Bingham 1983; Woodfield and Bingham 1995), and higher yields could be achieved by the long-term accumulation of favorable additive alleles in blocks. However, a large portion of the genetic variation for forage yield has been explained by non-additive components (Rowe and Hill 1981). In fact, Kehr and Gardner (1960) and Dudley et al. (1969) found that approximately two-thirds of the genetic variance for forage yield in alfalfa was non-additive. Thus, vast improvements could be made quickly by capitalizing on non-additive gene action, and this could be done most effectively by selecting for heterotic hybrid combinations.

Germplasm that differs genetically from cultivated alfalfa could potentially provide sources of new favorable alleles for chromosome regions in which complementation is low or absent in current cultivars. Alfalfa is distributed worldwide and grows in highly contrasting environments (Rumbaugh et al. 1988). This extensive geographical adaptation promotes genetic variation and gives breeders the possibility of using highly diverse gene pools. Between 1850 and 1947 several diverse gene pools were introduced to the USA in the form of nine accessions from different regions of the world: *M. falcata, M. varia*, *M. sativa* Ladak, Turkistan, Flemish, Chilean, Peruvian, Indian and African. These nine accessions make up the pedigrees of all North American alfalfa cultivars, although their genetic contribution varies (Barnes et al. 1977). Kidwell et al. (1994a) evaluated *Medicago* accessions representing these original nine germplasm sources for molecular marker diversity and found that only two sources, *M. falcata* and Peruvian, formed distinct clusters with respect to the other seven accessions. These distinct germplasms are a potential source of novel favorable alleles that could be used to improve alfalfa cultivars; however, the relationship of these sources to modern cultivars has not been tested.

Although utilizing diverse germplasm may appear attractive, data from previous studies have shown that the correlation between genetic diversity and heterosis is not consistent and depends on the genetic background of the material under evaluation (Bonierbale et al. 1993; Cress 1966; Moll et al. 1962; Paterniani and Lonquist 1963). Indeed, synthetic alfalfa populations developed on the basis of molecular marker diversity showed no consistent relation between diversity and forage yield (Kidwell et al. 1999). These findings indicate that unadapted germplasms should be evaluated in the field before being selected as potential donors of favorable alleles.

In the investigation reported here, we used molecular markers to study population genetic diversity among *M. sativa* ssp. *sativa* Peruvian, *M. sativa* ssp. *falcata* WISFAL, and three alfalfa cultivars. We also tested the potential of a novel Peruvian × WISFAL population to complement cultivated alfalfa by evaluating this germplasm in hybrid combination with commercial cultivars.

Materials and Method

Plant material

Seeds of *Medicago sativa* ssp. *sativa* Peruvian (PI 536535) were obtained from the Western Regional Plant Introduction Station in Pullman, Washington, USA (Melton et al. 1990). Seeds of *M. sativa* ssp. *falcata* WISFAL (PI 560333) were obtained from E.T. Bingham (University of Madison, Wisconsin, USA; Bingham, 1993). Sixteen plants each of *M. sativa* ssp. *falcata* WISFAL (Fal) and *M. sativa* ssp. *sativa* Peruvian (Peru) were randomly selected and organized into pairs for crossing, generating a total of 32 F_1 progenies including reciprocal combinations (PF and FP). Seed produced by reciprocal crosses were kept separate throughout the experiment. Three F_1 plants from each PF and FP combination and the parental plants (Peru and Fal) were crossed with three different cultivar testers—Ciba 2444 (C, obtained from ABI-Alfalfa), 54 V54 WY 9877 (Pi, obtained from Pioneer) and Rebound (R,, obtained from Forage Genetics). Cultivar testers were always used as females. Equal numbers of seeds were taken from each cross combination [48 for F_1 plants × testers and 16 for parent plants (Peru or Fal) × tester] and bulked separately for each tester. Parental plants were random-mated within the Peru and Fal groups and their seeds bulked to form the Peru and Fal seed sources. A three-way hybrid among the testers [(Pi × R) × C] was also produced. Plants were grown under greenhouse conditions, and all crosses were made by hand.

Field evaluation

Parents (Peru and Fal), F_1s (PF and FP), parents × testers, F_1s × testers, testers and the tri-tester hybrid were evaluated for forage yield for two seasons in two locations: Carillanca Research Station (lat 38°41′S, long 72°25′W) located in Temuco, Chile, and Arlington, Wis., USA (lat 43°20′N, long 89°23′W). The experimental unit consisted of 2-m row plots seeded with 100 seeds. To reduce the variable effects of adjacent plots, we planted a border row between each entry. Rows were spaced 0.2 m and 0.3 m apart in Temuco and Arlington, respectively. Seeds were planted in a nursery in October 1999, and approximately 1 month after germination plants were transplanted to the field in Temuco. Seed were directly planted to the field in April 2000 in Arlington. Irrigation was applied in both locations when it was necessary. Four replicates of each treatment were evaluated at each location using a randomized complete block design. Forage was harvested twice in both locations for the first season. For the second season, forage was harvested three and five times in Arlington and Temuco, respectively. The top growth was hand harvested from the central meter in Wisconsin and from the entire 2-m plot in Temuco. Forage samples were dried and then weighed. Analyses of variances (ANOVA) were performed to examine variation among genotypes for total dry matter (TDM). Seasons were analyzed separately. Variances were analyzed using the Mixed model from SAS (SAS 2001). Populations and locations were considered fixed effects in the model. Replications were considered to be a random effect. Least square means (lsmeans) were estimated for each population in both seasons (SAS 2001). Lsmeans were compared by the least significant difference (LSD) test (Sokal and Rohlf 1995). Mid-parent heterosis was measured as the deviation of hybrids from the average of parental germplasms, and significance levels were calculated using linear contrasts from the ESTIMATE command of SAS (2001).

DNA preparation, restriction fragment length polymorphism and simple sequence repeat analysis

Apical meristems and young leaves were collected from 16 plants each of Fal, Peru, Pi, R and C, and total genomic DNA was extracted from individual plants as described by Kidwell and Osborn (1992).

DNAs were digested separately with *Eco*RI and *Hin*dIII (Promega, Madison, Wis.) restriction enzymes. DNA digestion, gel electrophoresis, Southern blotting and hybridization conditions were carried out as previously described (Kidwell et al. 1994a). Southern blots were hybridized with 37 restriction fragment length polymorphism (RFLP) probes from a previously described set (Kidwell et al. 1994a). Two pairs of simple sequence repeat (SSR) primers, MTLEC2A and AFctt1 (Diwan et al. 2000), were used to amplify DNA samples by PCR using reaction mixes (Seah et al. 1998) and thermal cycling conditions (Diwan et al. 2000) described previously. PCR products were separated in a standard DNA sequencing gel containing 5% polyacrylamide, 7 *M* urea, and 1× TBE, at 60 W constant power.

Molecular data collection and analysis

RFLP and SSR DNA fragments that were polymorphic among genotypes were scored as present or absent using a binary code and then combined in a single data matrix. A principal component analysis (PCA) was performed on the simple correlation matrix computed from the data matrix (Joliffe 1986) using NTSYSPC 2.11A (Rolf 2000).

To infer the structure of the *M. sativa* germplasm, we analyzed the molecular marker data using a model-based clustering method developed by Pritchard et al. (2000). This model assumes the existence of *K* subpopulations (where *K* may be unknown), and each of these subpopulations is characterized by a set of allele frequencies at each locus. Individuals in the sample are assigned probabilistically to a subpopulation, or jointly to two or more subpopulations if their genotypes indicate that they are admixed. The number of subpopulations (or clusters) present in the *M. sativa* germplasms was inferred utilizing a Bayesian approach. Germplasm origin information was not included in the model. Thus, individuals were assigned to specific clusters based only on their genetic make-up. Due to the tetraploid nature of alfalfa, determining the genotype at RFLP and SSR marker loci is nearly impossible. For this reason, each class of genotypes was considered to be a haplotype (Pritchard et al. 2000). A prior distribution of *K* [Pr(*X*|*K*), where *X* denotes the genotypes of the sampled individuals] was estimated using the approximation developed by Pritchard et al. (2000), and the inference of *K* was made on the posterior distribution Pr(*K*|*X*). Because alfalfa populations and cultivars may have been developed by intermating several germplasms, the analyses were done using the admixture ancestry and correlated allele frequency models. The admixture model allows for mixed ancestry and assumes that a given individual *i* has inherited some fraction of its genome from ancestors in population *k*. It is important to note that the admixture model may not be formally correct when DNA markers are scored as dominant alleles. However, Falush et al. (2003) suggest that estimates based on admixture models may be reasonably unbiased if many loci are used in the analysis. The correlated allele frequency model assumes that population allele frequencies are not independent. Thus, frequencies among subpopulations are likely to be similar due to migration or shared ancestry (Falush et al. 2003). Correlations among subpopulations were determined using a modified Wright's F_{ST} (Falush et al. 2003). F_{ST} has been defined as the correlation between random gametes within subpopulations relative to gametes of the total population (Wright 1951, 1965). Traditionally, F_{ST} has been estimated as a single value that summarizes the average deviation of a collection of subpopulations away from the mean. The Falush et al. (2003) new *F* model is based on the existence of a P_A parameter, which records the allele frequencies in a hypothetical ancestral population. It assumes that the *K* subpopulations present in the sample have each undergone independent drift away from the ancestral population. Thus, each *K* subpopulation will have its own *F* estimate. The numerical values of the new *F* estimates are similar to F_{ST}, and larger values are inferred to represent the reduction of heterozygosity that each population has suffered because of genetic drift.

K, *F*, and *Q* plots (where *Q* denotes the estimated membership coefficients for each individual in each population) and allele frequency divergence were computed using STRUCTURE2 (Falush et al. 2003). All analyses were performed setting the ploidy level to tetraploid. The results were obtained using a burn-in period of 50,000 iterations and a collecting data length of 10^6 iterations.

Results

Population genetic diversity

A total of 212 polymorphic fragments were scored across the 79 evaluated individuals (191 RFLPs and 21 SSRs). Results from PCA of all individuals are presented in Fig. 1. The first, second, and third principal components (PC1, PC2, and PC3) accounted for 34.4%, 9.3% and 2.6% of the total variation detected among individuals, respectively. Fal germplasm was clearly separated from all of the other germplasms. Of the remaining germplasms, Peru individuals were somewhat separated from the cultivars, but individuals from the three cultivars appeared to be well interspersed (Fig. 1). Since PCA provides poor discrimination for closely related groups, it gave limited information on the genetic relationships among Peru, Pi, C and R.

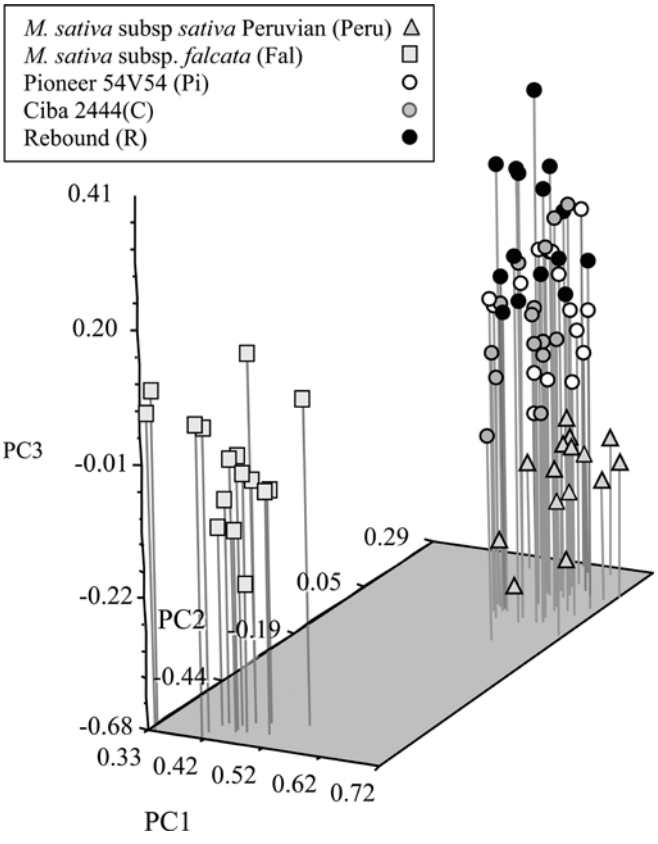

Fig. 1 Genetic relationships among individuals from five different *Medicago sativa* populations based on the first three principal components (*PC1*, *PC2*, *PC3*) derived from multivariate analysis of RFLP and SSR data

To obtain more information on the relationships of the germplasms, we performed a population structure analysis (Pritchard et al. 2000). Table 1 summarizes the Pr($X|K$) estimates for K=2,⋯, 5 and corresponding values of Pr($K|X$) for a uniform prior on K=2,⋯, 5. From the estimates of the posterior probability of K, it is clear that K=4 (four subpopulations or clusters) is substantially better than other values of K. Given these results, later analyses were done using K=4. Proportions of membership of each germplasm (Fal, Peru, Pi, R and C) to each of the four clusters are shown in Fig. 2. Fal, Peru and R belonged almost entirely to different clusters (cluster 3, 1 and 4, respectively). Pi and C were assigned to a common cluster, but some individuals showed high membership to the other clusters. Allele frequency divergence estimates (Table 2) agreed with the graphical representation created using PCA. Clusters 1, 2 and 4, including mostly Pi, R, C and Peru, were closely related and clearly diverged from cluster 3, including mostly Fal individuals. The highest divergence was obtained between cluster 1 (Peru) and cluster 3 (Fal).

F estimates (equivalent to F_{ST}) varied across germplasms. Cluster 3, which included mainly Fal individuals, showed the highest F (0.4637). This value was surprisingly high given the reproductive biology and the ploidy level of alfalfa. Clusters 1 and 4 (which mostly included Peru and R, respectively) had moderate F estimates (0.1490 and 0.1427, respectively). Cluster 2, which included Pi and C, showed an inferred F close to zero (0.0780), indicating a low level of genetic drift for these two alfalfa cultivars.

Forage yield evaluation

The potential of the Fal, Peru, FP and PF populations to complement cultivated alfalfa was evaluated by comparing forage yields of these populations, the three cultivar testers and testcrosses between them. Most plants of cultivar Pi died at the seedling stage in Arlington for unknown reasons; therefore, it was considered as missing data for both seasons at this location. ANOVA (Table 3) revealed that the population component was the only significant source of variation in Season 1. Thus, for Season 1, population means were estimated for data combined from both locations (Fig. 3a). In general, Peru yielded significantly higher than Fal, both as populations per se and as hybrids with alfalfa cultivars. Significant mid-parent heterosis was found for PF and FP when compared with Peru and Fal (P-value 0.037 and 0.014, respectively). The hybrid between Peru and R was significantly higher yielding than most cultivars and produced the highest yield of all the entries. The PF and FP populations yielded more than the parental germplasms (Peru and Fal) and performed as well as the cultivated types when hybridized with Pi, C or R.

Season 2 was characterized by the presence of a highly significant location × population interaction (Table 3). Although both locations, Temuco and Arlington, are characterized by cold winters and warm summers, climate conditions are more extreme in Arlington. Arlington winters are snowy, and average temperatures during the December–March period are below or very close to 0°C (http://mcc.sws.uiuc.edu/Temp/WI/470308_tsum.htlm1). Because of this location × population interaction, populations were analyzed separately for each location (Fig. 3b). The general pattern of forage yield did not change greatly from Season 1; however, some specific differences were observed. In Season 1, Peru and testcrosses with Peru had higher yields than Fal and testcrosses with Fal at both locations, whereas this yield difference was observed only in Temuco in Season 2. Peru is a non-dormant germplasm, and its relative lower yield in Arlington in Season 2 is probably related to less winter-hardiness in that location. Another difference in Season 2 was that FP yielded significantly more than PF in Arlington but not in Temuco. This difference also was observed in comparing estimates for mid-parent heterosis. In Temuco, both PF and FP showed significant mid-parent heterosis when compared to Peru and Fal (P-value=0.037 and 0.014, respectively. However, in Arlington, only FP showed significant mid-parent heterosis (P-value=0.013). As observed in Season 1, the PF and FP testcross populations performed as well as the cultivated types; however, there was a slightly greater yield for PF and FP crossed with R. This yield pattern was observed in both locations, although yields overall tended to be higher in Temuco.

Table 1 Probabilities and variances from inferring the number of clusters (K) for five *Medicago sativa* germplasms

K	ln $P(X/K)$[a]	var[ln$P(X/K)$][b]	$P(K/X)$[c]
2	−6784.4	221.4	3.74E–85
3	−6679.5	352.1	1.35E–39
4	−6590	489.1	0.9994
5	−6597.4	655.6	0.0006

[a]Natural logarithm of estimated probability of the data
[b]Variance of ln $P(X/K)$
[c]Posterior probability of K

Table 2 Allele frequency divergence[a] among clusters computed using point estimates of P (where P denotes the unknown allele frequencies in all populations)

Cluster[b]	Cluster[b]			
	1	2	3	4
1	–			
2	0.055	–		
3	0.410	0.355	–	
4	0.075	0.055	0.360	–

[a]Divergences were estimated using the Kullback-Leibler distance algorithm
[b]Cluster 1 includes mostly *M. sativa*ssp. *sativa* Peruvian; cluster 2 includes mostly Pioneer 54 V54 and Ciba 2444; cluster 3 includes mostly *M. sativa* ssp. *falcata* WISFAL; cluster 4 includes mostly Rebound

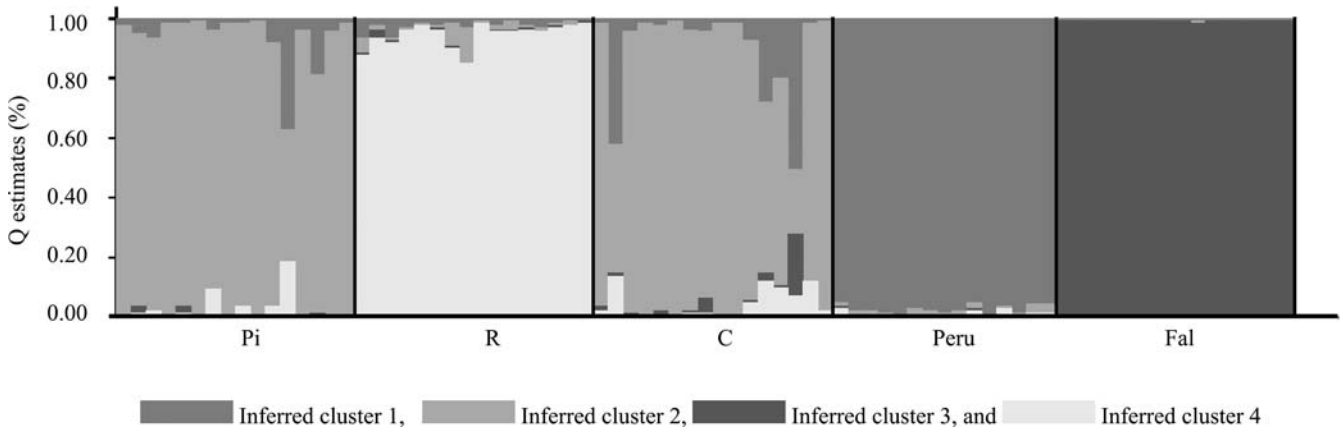

Fig. 2 Bar plot of Q estimates for five *M. sativa* germplasms, where *Q* denotes the estimated membership coefficients for each individual in each of four inferred clusters 'represented' by four degrees of shading. Each individual is represented by a *column* broken into up to four *segments* with the segment's length proportional to the degree of membership in each of four inferred clusters. The five sections delimited by the *x-axis* correspond to predefined germplasms: *Pi* Pioneer 54 V54, *R* Rebound, *C* Ciba 2444, *PeruM. sativa* ssp. *sativa* Peruvian, *FalM. sativa* ssp. *falcata* WISFAL

Discussion

The *M. sativa* ssp. *falcata* WISFAL germplasm (Fal) is clearly different from the other alfalfa germplasms we analyzed, as demonstrated by PC and population structure analyses, a result which is in agreement with previous reports (Kidwell et al. 1994a; Musial et al. 2002). Fal was developed by backcrossing tetraploidy into yellow-flowered, diploid *M. sativa* ssp. *falcata* accessions using 2*n* eggs (Bingham 1990), and although different diploid plants were used in each backcrossing cycle, the number of plants used in the last three backcrosses was small (five to six individuals). Thus, the genetic distinctiveness of Fal may be due, in part, to genetic drift, which was also indicated by the large *F* estimates in our study. However, another molecular marker study including several diploid *M. sativa* ssp. *falcata* accessions also found that this subspecies tended to group in a distinct cluster (Brummer et al. 1991). Genetic distinctiveness has not been observed for some tetraploid *M. sativa* ssp. *falcata* germplasms (Crochemore et al. 1998; Riday et al. 2003), but this could be due to subspecies admixture, since variegated or purple flowers have been observed within tetraploid *M. sativa* ssp. *falcata* germplasms (Brummer et al. 1999; Crochemore et al. 1998; Lesins and Lesins 1964).

Our analysis also identified Peru as a distinct germplasm but one genetically closer to the three modern cultivars than Fal. In a previous study Kidwell et al. (1994a) found that the Peru germplasm was genetically different from the other original germplasm sources introduced to North America, but modern alfalfa cultivars were not included in their analysis. Our results, particularly those from the population structure analysis, showed that Peru is different from the three modern alfalfa cultivars. Peruvian germplasms originated in Peru and are the descendants of alfalfas introduced to this South American country more than 200 years ago (Barnes et al. 1977). The genetic distinctiveness of Peru could be explained in part by this geographic isolation.

Population structure analysis also showed that R is somewhat distinct from the other cultivars. Most alfalfa cultivars are genetically broad-based synthetics developed by randomly intermating several selected parents and advancing their offspring through several open-pollinated generations (Hill et al. 1988). Therefore, genetic distinctiveness among cultivars could be explained by genetic differences among the parental plants (ancestry), number of selected plants (genetic drift), artificial selection for specific traits, or a combination of these factors. R showed a higher *F* estimate than cluster 2 (mostly including Pi and C), suggesting a higher degree of genetic drift in this cultivar. In addition, pedigree data indicate differences in the amount and type of germplasms used to develop these cultivars (Barnes et al. 1977; The North American Alfalfa Improvement Conference, http://www.naaic.org/).

Table 3 Analysis of variance of total dried matter harvested in Seasons 1 and 2

Source of variation	*df*	Season 1			Season 2		
		Sum of squares	*F*	*P*	Sum of squares	*F*	*P*
Location	1	22.39	3.27	0.1196	17.29	2.15	0.1906
Population	19	143.98	5.50	<.0001	1369.54	10.38	<0.0001
Location × population	19	32.19	1.23	0.2467	415.45	3.32	<0.0001
Rep (Location)	6	42.67	5.17	0.001	48.40	1.16	0.3322
Error	110	151.43			763.86		
Total	155	392.66			2614.54		

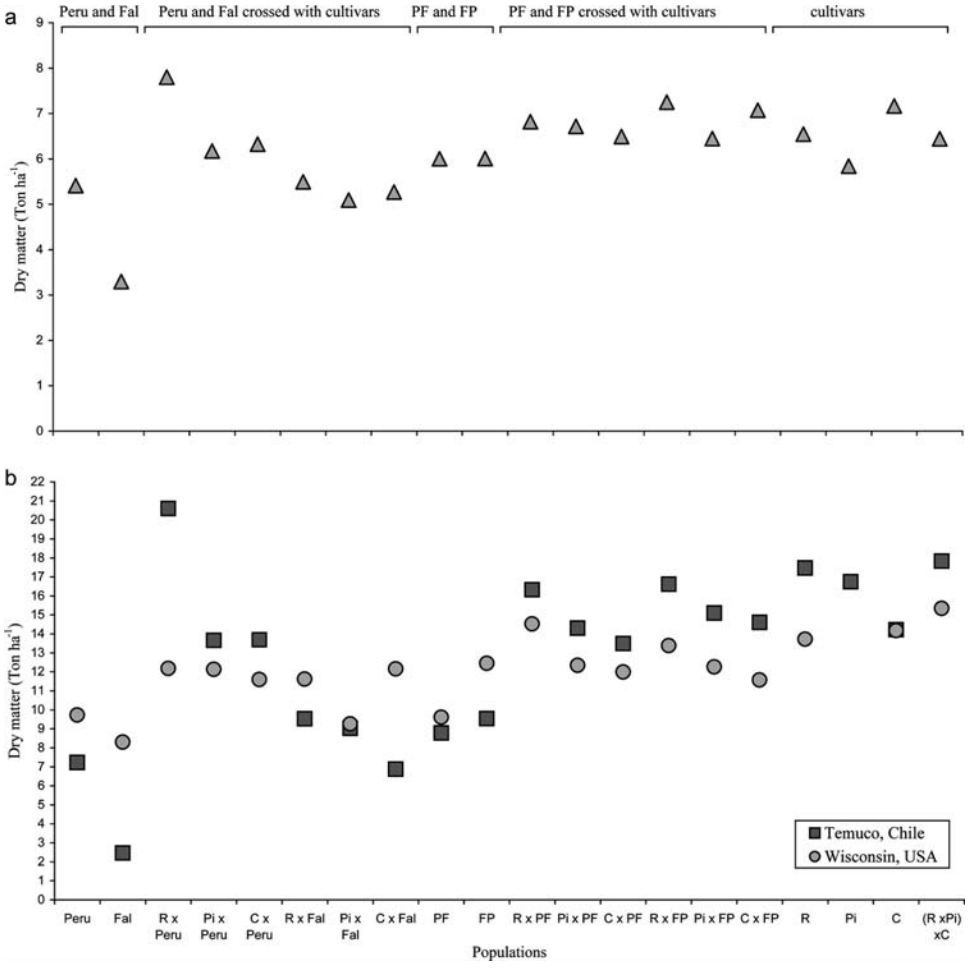

Fig. 3a,b Total dry matter (lsmeans in Ton ha^{-1}) harvested from *M. sativa* populations. *PeruM. sativa* ssp. *sativa* Peruvian, *FalM. sativa* ssp. *falcata* WISFAL, *PF*, *FP* Peruvian × WISFAL F_1s (including reciprocal crosses), *Pi* cv. 54 V54 WY 9877 obtained from Pioneer, *C* cv. Ciba 2444 obtained from ABI-alfalfa, *R* cv. Rebound obtained from Forage Genetics. Since there was no significant population × location interaction in Season 1 (**a**), populations lsmeans were estimated by combining the data for both locations. In Season 2 (**b**), each location is shown separately. Data for Pi was missing in Wisconsin. LSD (**a**): 1.16 Ton ha^{-1}; LSD (**b**): 2.61 Ton ha^{-1}

Our results from field evaluations suggest that Fal and Peru have potential as donors of favorable alleles to improve forage yield in cultivated alfalfa. Hybrids between Peru and Fal germplasms showed clear mid-parent heterosis. A similar result was reported previously in evaluations of hybrids between other accessions of *M. sativa* ssp. *sativa* and *M. sativa* ssp. *falcata* subspecies (Riday and Brummer 2002; Sriwatanapongse and Wilsie 1968; Waldron 1920; Westgate 1910). A heterotic tendency, although not significant, was also observed when Peru and Fal were independently hybridized with the cultivars. Together, these observations suggest that Peru and Fal may have different alleles that could complement those in current alfalfa cultivars.

Hybrids between PF and FP populations and the cultivars yielded as much as the cultivars per se and the three-way hybrid among cultivars. PF and FP are hybrid populations derived from crosses of heterogeneous germplasms (Peru and Fal). Therefore, the combination of chromosome blocks responsible for yield increase will segregate as soon as they are sorted into gametes through meiosis, and this may have reduced the complementary effect of the PF and FP testcrosses. In addition, it is very likely that Peru and Fal have undesired alleles for some agronomic traits. Thus, the concentration of favorable complementary factors and the elimination of unwanted loci will be necessary to realize the complementary capacity of the Peru × Fal population with modern cultivars.

The observation of forage yield differences between the reciprocal crosses PF and FP in Arlington-Season 2 raises the question of whether this difference could be due to cytoplasmic effects. PF and FP share the same nuclear genomic constitution; however, cytoplasmic components should differ because chloroplasts are largely paternally transmitted and mitochondria are maternally transmitted in alfalfa (Forsthoefel et al. 1992; Rusche et al. 1995; Zhu et al. 1993). A cytoplasmic effect was not observed for the testcrosses with PF and FP, which had different paternal lineages (Fal for cultivars × PF and Peru for cultivars × FP). Thus, the yield difference between PF and FP in Arlington-Season 2 may have been due to maternally transmitted factors, perhaps through their effect on winter-hardiness, since no difference was observed before the winter in Season 1. Further research should clarify the potential effect of cytoplasms on forage yield and winter-hardiness.

Our results also show that there were differences in combining ability among cultivars. Cultivar R combined well with PF and FP in all locations and years and produced the best forage yields in hybrid combination with Peru in Seasons 1 and 2 in Temuco. Molecular data

suggest that this cultivar is genetically more distant from Peru than the other cultivars. Thus, the better combining ability of this cultivar with PF, FP and Peru could be explained by the presence of different favorable alleles among these germplasms. This explanation is also supported by pedigree data in which Peru shows no contribution to the development of R (The North American Alfalfa Improvement Conference, http://www.naaic.org/).

Conclusions

The results from this study indicate that *M. sativa* ssp. *falcata* WISFAL and *M. sativa* ssp. *sativa* Peruvian germplasms are genetically diverse from the evaluated modern alfalfa cultivars. This genetic distinctiveness suggests that these sources may have novel alleles that could complement alfalfa, and the results from forage yield evaluations further support this hypothesis. If Peru and Fal contain favorable alleles at different loci, it may be possible to concentrate them in an improved population derived from the Peru × Fal hybrids. We have initiated a recurrent selection program by intermating individuals from this novel Peru × Fal population and evaluating testcross progenies. This strategy should allow us to reduce the frequency of undesirable alleles and concentrate favorable complementary factors governing forage yield of cultivated alfalfa.

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