Theor Appl Genet (2007) 115:859–867
DOI 10.1007/s00122-007-0614-1

ORIGINAL PAPER

Analyses of a multi-parent population derived from two diverse alfalfa germplasms: testcross evaluations and phenotype–DNA associations

I. J. Maureira-Butler · J. A. Udall · T. C. Osborn

Received: 19 April 2007 / Accepted: 8 July 2007 / Published online: 4 August 2007

Abstract In a previous study, we showed that the genetic variation present in the *Medicago sativa* subsp. *sativa* Peruvian and *M. sativa* subsp. *falcata* WISFAL germplasms could be used to improve forage yields when favorable alleles were recombined and used in hybrid combination with cultivated alfalfa. In this paper, we present testcross forage yield and fall growth data for two seasons of a C0 population generated after intermating the Peruvian × WISFAL population for several generations. In addition, we conducted marker-trait association analysis as an attempt to identify Peruvian and WISFAL genomics regions affecting the targeted traits. Five and seven genomic regions were found significantly associated with forage yield and fall growth, respectively. In the case of fall growth, alleles from both accessions were positively associated with plant height. However, more alleles from WISFAL were positively associated with forage yield than from Peruvian. WISFAL is known for its winter hardiness and genomic regions with large effects on winter survival may have masked the effect of forage yield from Peruvian. The fact that most of the genomic regions discovered in this study have been previously associated with traits involved in winter hardiness validates our findings and suggests that associations between DNA fragments and agronomic traits can be detected without the necessity of developing bi-parental mapping populations.

Communicated by T. Lübberstedt.

I. J. Maureira-Butler (✉) · J. A. Udall · T. C. Osborn
Plant Breeding and Plant Genetics Program
and Department of Agronomy,
University of Wisconsin, Madison, WI 53706, USA
e-mail: imaureira@inia.cl

I. J. Maureira-Butler
Agri aquaculture Nutritional Genomic Center,
Plant Biotechnology Unit, INIA Carillanca,
P.O Box 58-D, Temuco, Chile

Present Address:
J. A. Udall
Brigham Young University,
Provo, UT 84602, USA

Present Address:
T. C. Osborn
Seminis Vegetable seeds (A Division of Monsanto),
State Highway 16, Woodland, CA 95695, USA

Introduction

Genetic improvement through recurrent selection has been a critical component of yield increases in crop plants. Probably, the most impressive examples of genetic improvement are found in corn and sorghum breeding, in which average genetic gains per year are 1.42–1.78% (Duvick 1984) and 1.3% (Miller and Kebede 1984), respectively. Genetic improvement has also been achieved in forage crops; for instance, genetic gains of 6% and 5% per decade have been observed in white clover (Woodfield and Caradus 1994) and rye grass (Van Wijk and Reheul 1991), respectively. By using phenotypic recurrent selection, alfalfa breeders have been able to improve a wide number of traits, such as disease and insect resistance (Barnes and Hanson 1971; Heisey and Murphy 1985; Bray and Irwin 1989; Salter et al. 1994; Elden and Elgin 1987), salt tolerance (Dobrenz et al. 1993), and forage quality (Demment et al. 1986; Sumberg et al. 1983). However, improvement of forage yield has been slow and gains per year have been barely higher than zero [estimated to be 0.18% in both Holland and Bingham (1994) and Hill and Kalton (1976)].

Alfalfa cultivars are genetically broad-based synthetics traditionally developed using intra-population breeding methodologies, in which selected parents are randomly intermated and their offsprings advanced through several generations of open-pollination (Busbice 1969; Hill and Elgin 1981; Hill et al. 1988). This methodology has produced more persistent and disease resistant cultivars (Hill et al. 1988; Rotili et al. 1999), but it has not significantly exploited the variation present within the species to improve forage yields. Rowe and Hill (1981) noted that if there is evidence for non-additive gene action controlling yield, some form of inter-population improvement should be superior to most intra-population improvement methods. Although additive variation has been shown to be important for alfalfa forage yield (Pfeiffer and Bingham 1983; Woodfield and Bingham 1995; Kimbeng and Bingham 1998a, b, 1999), genetic variance studies have estimated that the non-additive component accounts for approximately two-thirds of the total genetic variation (Kehr and Gardner 1960; Dudley et al. 1969). Thus, forage yield could be improved using an inter-population recurrent selection strategy (Brummer 1999; Segovia-Lerma et al. 2004).

Alfalfa is an allogamous tetraploid species with polysomic inheritance, which creates higher levels of genetic complexity than that observed in diploid individuals (Stanford 1951; Quiros 1982). This additional complexity may have contributed to the limited yield gains of selection in alfalfa when compared to the yield gains of other diploid crops. Bingham et al. (1994) proposed that yield in tetraploid alfalfa could be increased by accumulating chromosome blocks containing favorable alleles in repulsion linkage phase. Their model helps to explain a number of genetic phenomena, such as severe inbreeding depression and progressive heterosis (Jones and Bingham 1995; Pfeiffer and Bingham 1983; Dunbier and Bingham 1975; Groose et al. 1989; Scotti et al. 2000), and provides a theoretical framework to account for the high level of non-additive variation observed in quantitative studies. Although chromosome combinations achieved by hybridizing heterotic parents will be rapidly lost after chromosome sorting during meiosis, inter-population recurrent selection could be used to select for complementary interactions between two populations and then commercially exploit it through a hybrid population once sufficient complementation has been achieved.

In a previous study, we suggested that the variation present in a population derived from hybridizing *Medicago sativa* subsp. *sativa* Peruvian and *M. sativa* subsp. *falcata* WISFAL could be used to improve modern alfalfa cultivars (Maureira et al. 2004). We provided evidence that each of these genetically distinct germplasms contained favorable factors that could complement current alfalfa cultivars and suggested that combining them within an improved population could result in forage yield increases. Such favorable factors coming from the Peruvian and WISFAL germplasms could be associated with segregating molecular markers. Marker-trait associations would provide evidence of genomic regions containing quantitative trait loci (QTL) for forage yield and help to understand the genetic architecture of this complex trait. Although QTL mapping has allowed the discovery of regions affecting important traits in tetraploid alfalfa (Brouwer et al. 2000; Cao et al. 2005; Musial et al. 2005), the QTL detection has been done using bi-parental populations, which are not always available or compatible with alfalfa breeding programs (Osborn et al. 1998; Maureira and Osborn 2005). Obert et al. (2000) recently associated AFLP markers with resistance to downy mildew (*Peronospora trifoliorum* de Bary) by analyzing improved synthetic populations, which are commonly developed by alfalfa breeders. This illustrates the possibility of uncovering important genomic regions without developing bi-parental mapping populations that have typically been used for this type of genetic analysis (Brouwer and Osborn 1999; Robins et al. 2007a, b).

In this paper, we report hybrid forage yield and levels of fall growth for a novel population derived from the WISFAL-Peruvian population after several cycles of intermating. This population constitutes the cycle 0 (C0) of a recurrent selection program, in which selection will be based on the capacity to complement cultivated alfalfa through hybridization with cultivated testers. We also present marker-trait association tests in an attempt to identify WISFAL-Peruvian genetic factors controlling forage yield and fall growth in a hybrid testcross genetic background.

Materials and methods

Plant material

Sixteen plants each of *M. sativa* subsp. *falcata* WISFAL (Fal) and *M. sativa* subsp. *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) as previously described (Maureira et al. 2004). Three F_1 plants from each PF and FP cross were randomly selected and intercrossed at random with all other F_1 plants to generate the F_2 generation. A similar crossing scheme was used to develop the F_3 and F_4 generations. The F_3 generation was developed by random mating two selected F_2 plants from each F_1 plant to all other F_2 plants. The F_4 generation was developed by random mating one F_3 plant of each previously selected F_2 to all other F_3 plants. Sterile, weak, or sick plants were eliminated in each generation. A total of 131 F_4 plants constituted the C0 of our recurrent selection program. F_1 plants (PF and FP), the

parental plants (Peru and Fal) and the 131 C0 plants were test crossed with two different cultivar clone testers, MIII-17 and Lgdy-S1 (obtained from E.T. Bingham, University of Wisconsin, Madison, USA). MIII-17 is a selected male-sterile progeny from a cross of a Magnum III male-sterile plant × Blazer plant number 17. Blazer 17 has normal male- and female-fertility, and maintains male-sterility in crosses with male-steriles. Moreover, Blazer 17 was used as a parent of mapping bi-parental populations in previous studies (Brouwer and Osborn 1999). Lgdy-S1 is a selected S1 from the cultivar Legendary. Although Lgdy-S1 produces good pollen, it is essentially self-sterile (E.T. Bingham, personal communication). Cultivar testers were always used as females. Equal numbers of seeds were taken from each F_1 (PFs and FPs) and parental (Fal and Peru) cross combination and bulked separately for each clone tester. A hybrid between testers (MIII-17 × Lgdy-S1) was also produced. Plants were grown under greenhouse condition and all crosses were made by hand. Two cultivars, Ciba 2444 (obtained from ABI-Alfalfa) and Vernal (provided by Kevin Silveira, University of Wisconsin, Madison, USA), were used as controls because they represent cultivars that were once widely grown as a forage crop and are parents to many currently used cultivars.

Field evaluation and experimental design

Parents × testers, F_1s × testers, 131 C0 × testers, the hybrid between testers, and the two control cultivars were evaluated for forage yield and fall growth at Arlington, Wisconsin USA (lat 43°20′N, long 89°23′W). Forage yield was evaluated for two seasons and fall growth was measured at the end of the first season. The experiment was planted in a completely randomized split-plot design with two replications. The testers represented the main plots and the testcrosses, randomized within main plots, represented the subplots. The subplots were five rows wide, with 0.15 m between rows and 1.22 m long, and only a central 0.31 m strip across the plot was harvested. Five hundred seeds were planted per plot and were directly sowed into the field in May 2001. Irrigation was applied when necessary in the first season. Plots were visually inspected for the presence of diseases; however, no obvious symptoms were observed. Forage was harvested once the first season and three times the second season. Yield of the second season was measured as the cumulative dry matter weight of all three cuttings. Some individual plants contributing to plot yield in year one may not have contributed to the plot's yield during year 2 due to poor winter survival. Across years, no attempt was made to adjust forage yield on a per plant basis because such plant loss was intrinsic to each genotype and would have also occurred in a 'farmers field'. Forage samples were dried and then weighed. Fall growth was measured as the distance from the soil level to the top of the undisturbed plot on November 2001.

Statistical analysis of phenotypic data

Analyses of variances (ANOVA) were performed to examine variation among genotypes for total dry mater and fall growth. Variances were analyzed using the mixed model from SAS (SAS 2001). Testers and C0 testcrosses were considered fixed effects in the model. Replications were considered a random effect. Least square means (lsmeans) were estimated for each C0 line (SAS 2001). Lsmeans were compared by the least significant difference (LSD; Sokal and Rohlf 1995). Phenotypic correlations between forage yield and fall growth were performed using the CORR procedure from SAS (2001).

DNA preparation, RFLP and SSR analysis

Apical meristems and young leaves were collected from each C0 plant and the cloned testers, 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, WI, USA) restriction enzymes. Southern blots were produced and hybridized with 37 RFLP probes distributed across the alfalfa genome, as previously reported (Maureira et al. 2004). Two pairs of SSR primers, MTLEC2A and AFctt1 (Diwan et al. 2000), were used as described by Maureira et al. (2004).

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 (1/0). Thus, alleles were not be assigned to any fragments because segregation information of these sized fragments was not available. Each individuals genotype was assembled into a single data matrix. The C0 lsmean data obtained from forage yield and fall growth evaluations were combined with the genotype data matrix. The trait values of all individuals with the DNA fragment (1) were compared to those of all individuals without the fragment (0) by *F* tests. *F* tests were performed using the General Linear Model (GLM) from SAS (2001) and significant levels were empirically estimated by permutations (Churchill and Doerge 1994; Doerge and Churchill 1996). Permutations randomly shuffled trait values with respect to individual genotypes and marker-trait associations were performed with the permuted data sets, respectively. In order to generate stable critical values of significance, 10,000 permutations were carried out for each marker.

Markers that had a permutation significant level of <0.05 for the single marker analysis were selected and included in a multiple regression analyses. Multiple regression analysis was performed using the REG procedure with STEPWISE method selection from SAS (2001). A significant level of 0.05 was used for adding and keeping marker variables in the model.

Results

Phenotypic data

Analysis of variances for forage yield showed that the C0 testcrosses were significantly different for seasons 1 and 2; however, testers were significantly different only for season 2 (Table 1). Since the tester*C0 testcross interaction was non-significant, estimates of C0 testcross were averaged across testers (Table 1). The forage yield distribution is shown in Fig. 1a for season 2. Approximately, 9% of the C0 testcrosses were significantly higher than the Peru testcross and at least one C0 testcross significantly out yielded both the Fal and PF-FP F1 testcrosses.

Fall growth was measured as plant height at the end of season 1. Analysis of variance showed that both testers and C0 testcrosses were significant sources of variation (Table 1). Tester*C0 testcross interaction was not significant; thus, estimates were averaged across testers. Fall growth distribution is shown in Fig. 1b. About 60% of the C0 testcrosses were significantly taller than the Fal testcross and at least one C0 testcross outgrew the PF-FP F1 testcross. The phenotypic correlation between fall growth and forage yield was low and positive ($r = 0.17$, $P = 0.056$).

Molecular marker data

A total of 184 DNA fragments were analyzed for significant marker-trait associations using F tests. Nine and eleven of these fragments were significantly associated ($P < 0.05$, empirically estimate by permutation) with forage yield season 2 and fall growth, respectively (Table 2). Multiple regression models were used to eliminate spurious associations and markers weakly associated to the traits (Champoux et al. 1995; Sills et al. 1995). Five markers for forage yield and seven markers for fall growth were kept in the models using a significance level of $P < 0.05$, and these were considered significantly associated with the traits. Partial R^2s ranged from 3.8 to 7.7 and 3.4 to 8.2 for forage yield and fall growth, respectively (Table 2). All together, the markers account for 24.6 and 34.1% of the total phenotypic variation present for forage yield and fall growth, respectively. Putative alfalfa and *M. truncatula* linkage group positions are listed based on previously published map position of DNA fragments detected by the same probes (Table 2). Five and seven apparently unlinked factors appeared on four and five different linkage groups for forage yield and fall growth, respectively.

Discussion

Genetic diversity has always been the propeller of plant breeding and population improvement. Our C0 population represented two novel sources of alfalfa genetic diversity because it was derived from repeated intermatings of two unadapted germplasms, *M. sativa* subsp. *sativa* Peruvian × *M. sativa* subsp. *falcata* WISFAL. Utilization of novel variation is not a simple task since unadapted germplasm can bring undesired genetic factors that lower yields or diminish quality. However, our testcross data suggested that variation coming from the recombination of these unselected germplasms could improve forage yields. Others have also suggested that crosses between diverse alfalfa germplasms, especially those involving *M. sativa* subsp. *sativa* and subsp. *falcata* could improve forage yield (Maureira et al. 2004; Riday and Brummer 2004; Segovia-Lerma et al. 2004; Riday and Brummer 2002; Sriwatanapongse and Wilsie 1968; Waldron 1920). Thus, *M. sativa* subsp. *sativa* and subsp. *falcata* are valuable sources of genetic diversity for improving forage yield in alfalfa cultivars.

The phenotypic distributions of our C0 testcrosses showed a typical shape for quantitative traits, suggesting that a number of loci were segregating for forage yield and fall growth. Moreover, the presence of transgressive segregation indicated the existence of favorable variation in both original germplasms. The FP/PF hybrids represent unimproved germplasm that in hybrid combination compared

Table 1 Significance of fixed effects from ANOVA for forage yield seasons 1 and 2 and fall growth (measured as plant height)

Source of variation	Forage yield season 1		Forage yield season 2		Fall growth	
	F value	*P* value	*F* value	*P* value	*F* value	*P* value
Tester	3.55	0.2596	36.59	0.0263	128.75	0.0045
C0 testcross	1.38	0.0131	1.91	<0.0001	3.04	<0.0001
Tester × C0 testcross	1.14	0.1826	1.01	0.4769	0.94	0.6397

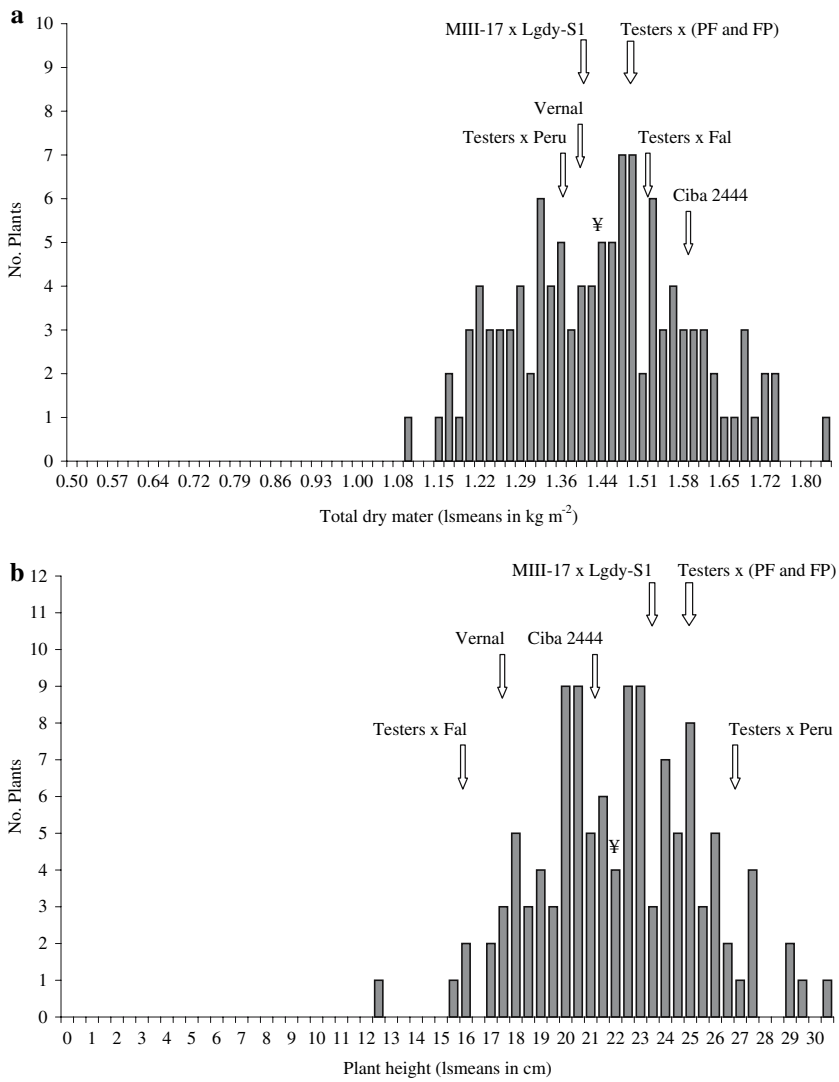

Fig. 1 Frequency distributions of C0 testcross populations season 2 (combined for both testers, MIII-17 and Lgdy-S1) for total dry matter **a** and plant height measured in the fall (**b**). ¥ refers to the population mean. Peru *Medicago sativa* ssp. *sativa* Peruvian, Fal *M. sativa* ssp. *falcata* WISFAL, Vernal and Ciba2444 are alfalfa cultivars. PF and FP are reciprocal crosses between *Medicago sativa* ssp. *sativa Peruvian* and *M. sativa* ssp. *falcata* WISFAL. LSD (**a**), 0.28 LSD (**b**): 4.82

exceptionally well to hybrids of older cultivars suggesting tremendous potential of this un-improved material. The transgressive phenotypes observed in some testcrosses suggest that novel combinations of favorable alleles may have contributed to the higher values of forage yield and fall growth than the parental germplasms.

The genetic component of phenotypic variation was estimated by marker-trait associations and we identified several Peruvian alleles with a favorable effect on forage yield and fall growth. Peruvian alleles detected by marker UWg328 were positively associated with an increase forage yield and explained more than 4% of the phenotypic variation. Peruvian alleles were also favorably associated with six genomic regions for fall growth and explained a total of ~28% of the phenotypic variation. The effects of one novel genomic region may have had an impact on both traits. While Peruvian alleles of UWg328 appeared to increase in forage yield, markers UWg328 and UWg96 were previously found to be tightly linked and had alleles that were positively associated with fall growth (Brouwer et al. 2000).

Four other regions were strongly associated with forage yield and the yield increase appeared to be due to the WISFAL allele. This result was unexpected and somewhat contradicted previous reports where Peruvian showed positive heterotic yield responses (Segovia-Lerma et al. 2004; Maureira et al. 2004). Our experiment was grown in an area characterized by extreme winter conditions and the observed WISFAL-marker bias could be explained by differences in winter survival between germplasms (Brouwer et al. 2000, 1998; Bingham 1993). Previous studies have identified genomic regions controlling freezing and winter injury across the entire alfalfa genome (Brouwer et al. 2000; Cao et al. 2005) and the strong effect of these genetic factors may have masked the effect of favorable Peruvian variation. In fact, most DNA markers we found associated with forage yield were either directly or indirectly associated with winter hardiness traits (Brouwer

Table 2 DNA markers significantly associated with forage yield season 2 and fall growth after single-marker analysis and multiple regression

DNA marker	*P* value[a] for single marker analysis	Partial R^2s Multiple reg[b]	Germplasm origin of marker[c]	M.truncatula chromosome/ linkage group[d]	Linkage group position[e]		
					Echt et al. (1994)	Tavoletti et al. (1996)	Brouwer and Osborn (1999)
Forage yield'							
Vg2c1	0.048	NS	NR	2	NU	unl	2
UWg96	0.031	NS	NR	1	3 and 5	3 and 5	3
UWg328	0.016	4.07	Peru, Fal (0)	1	1 and 3	1 and 3	NU
UWc59	0.020	NS	NR	4	5	5.	NU
Vg1h6	0.007	7.70	Fal (0)	8	NU	4	4
UWg35	0.044	3.78	Fal (1)	4	5	5	NU
Vg1g9	0.019	5.06	Fal (1)	4	NU	5	5
Hg2b8	0.039	NS	NR	4	NU	5	5
UWg169	0.019	3.98	Fal (1)	7	8	NU	8
Full model		24.59					
Fall growth							
Vg1h8_1	0.037	NS	NR	6	NU	7	1
Vg1h8_3	0.020	4.19	Peru (0)	6	NU	7	1
Vg2b11	0.038	3.40	Peru, Fal (0)	2	NU	3 and 6	2
UWc56	0.009	NS	NR	1	3	NU	NU
UWg96	0.043	4.87	Peru, Fal (1)	1	3 and 5	3 and 5	3
UWg295	0.013	3.43	Peru (0)	1	3	NU	NU
UWg138	0.034	6.25	Fal (1)	4	5	NU	NU
Vg2a2	0.048	NS	NR	4	NU	5	5
AFctt1_3	0.019	3.73	Peru, Fal (1)	7	NU	NU	8[6]
AFctt1_4	0.006	NS	NR	7	NU	NU	8
AFctt1_14	0.005	8.21	Peru, Fal (0)	7	NU	NU	8
Full model	34.08						

[a] *P* values were lower than the threshold estimated by 10,000 permutations

[b] Markers were included in the model using a stepwise selection method and a 0.05 significant level for entering and keeping variables in the model. *NS* not significant

[c] *Peru (0)*, the absence of the DNA fragment was associated with the increase of the phenotypic trait and was absent in all *Medicago sativa* subsp. *sativa* Peruvian original parental plants; *Fal (0)*, the absence of the DNA fragment was associated with the increase of the phenotypic trait and was absent in all *M. sativa* subp. *falcata* WISFAL original parental plants; *Fal (1)*, the presence of the DNA fragment was associated with the increase of the trait and was present in all *M. sativa* subp. *falcata* WISFAL original parental plants; *Peru, Fal (0)*, the absence of the DNA fragment was associated with the increase of the trait and segregated in both Fal and Peru original germplasms; *Peru, Fal (1)*, the presence of the DNA fragment was associated with the increase of the trait and segregated in both Fal and Peru original germplasms. *NR* not reported because it was NS in the model

[d] *M. truncatula* chromosome number was assigned by comparing common RFLP probes between Brouwer and Osborn (1999), Tavoletti et al. (1996), Echt et al. (1994), Robins et al. (2007a) and Kaló et al. (2000)

[e] Linkage group location of DNA fragments detected with these probes in previous studies. *NU* not used in the study, *unl.* marker was reported as unlinked

[6] Linkage group positions were determined by aligning Diwan et al. (2000) and Brouwer and Osborn (1999) linkage groups

et al. 2000; Cao et al. 2005). This observation suggests that more cycles of recombination and mild selection may be needed before the genetic contribution of the Peruvian germplasm could be accurately measured.

DNA markers from both *M. sativa* subsp. *sativa* Peruvian (non-dormant) and *M. sativa* subsp. *falcata* WISFAL (dormant) germplasms were favorably associated with fall growth. This finding was somewhat unexpected since WISFAL is fall dormant and winter hardy (Bingham 1993), and the WISFAL testcross was significantly shorter than the Peruvian testcross. However, this finding could be explained by the presence of favorable factors with small to moderate effects, which may be hidden by negative factors with large effects in the less favorable parent. Recombination exposes these hidden factors and allows breeders to pyramid them by selection and subsequent hybridization.

Our analysis showed that the phenotypic correlation between forage yield and fall growth was positive, although it was low. These results agree with findings observed in other segregating populations (Busbice and Wilsie 1965). However, our observed low correlation disagrees with the common practice of using fall dormancy, often evaluated by the amount of fall growth, as a predictor of winter hardiness (see Brouwer et al. 2000). Tall plants at the end of the season usually correlate with less winter survival and lowers yields in subsequent growing seasons (Smith 1961; Schwab et al. 1996; Brouwer et al. 1998). Comparisons of recent cultivars and studies of segregating F_2s have shown that winter hardiness and fall growth can be uncoupled (Barnes and Martin 1991; Busbice and Wilsie 1965; Daday and Greenham 1960; Brummer et al. 2000). Thus, our results with molecular markers suggest that favorable alleles in these populations can be revealed through one cycle of recombination and their complementary effects can improve hybrid population performance. Perhaps, non-dormant alfalfa cultivars with winter hardiness can be recovered with future cycles of recombination and selection.

Traditionally, discovery of genomic regions affecting important traits utilizes segregating populations developed from two parental genotypes, but the use of such traditional populations are limited in alfalfa. Alfalfa cultivars are typically synthetic populations with high levels of heterozygosity and heterogeneity. Selection of individual genotypes for mapping of traits in bi-parental populations would have sampled only a small portion of the resident variation within the original germplasm and may have reduced the odds of finding regions affecting the traits of interest. In our study, several genetic factors associated with the traits were segregating in both original Peruvian and WISFAL germplasms. By using sixteen genotypes from each Peruvian and WISFAL germplasm as progenitors of our C0 population, we were able to include a larger representative sample of the genetic variation present within these two germplasms, and this allowed us to uncover genomic regions that would have remained undetected by using a bi-parental population for mapping. The disadvantage to any multi-parental population is that the frequency of specific alleles may be too low to detect their effects.

Conclusions

The results of our study suggests that genetic variation present within our Peruvian × WISFAL C0 population could be used to complement the genetic variation in cultivated alfalfa for the improvement of forage yield. In addition, we were able to identify several genomic regions affecting both forage yield and fall growth, suggesting that genetic factors can be uncovered without the necessity of developing bi-parental populations typically used for these types of studies. Obviously, the regions we found to be associated with forage yield and fall growth need to be confirmed in future evaluations; however, the fact that several of these genomic regions were previously reported in an independent QTL mapping study (Brouwer et al. 2000; Cao et al. 2005) supported our findings. Since most of the positive effects on forage yield were detected coming from WISFAL, it remains to be seen if Peruvian will contribute positive effects in future cycles of recurrent selection.

Acknowledgments We thank E. T. Bingham and Amorntip Muangprom for comments on the manuscript. We also acknowledge Robert Vogelzang for technical assistance. Support was provided by a USDA Hatch grant from the University of Wisconsin, College of Agricultural and Life Sciences.

References

Barnes DK, Hanson CH (1971) Recurrent selection for bacterial wilt resistance in Alfalfa. Crop Sci 11:545–546

Barnes DK, Martin NP (1991) Varietal trials of selected farm crops. Minnesota Agric Exp Stn Rep 221-1991

Bingham ET (1993) Registration of WISFAL alfalfa (*Medicago sativa* subsp *falcata*) tetraploid germplasm derived from diploids. Crop Sci 33:217–218

Bingham ET, Groose RW, Woodfield DR, Kidwell KK (1994) Complementary gene interactions in alfafa are greater in autotetraploids than diploids. Crop Sci 34:823–829

Bray RA, Irwin JAG (1989) Recurrent selection for resistance to *Stemphylium versicarium* within the lucerne cultivars Trifecta and Sequel. Austr J Exp Agr 29:189–192

Brouwer DJ, Osborn TC (1999) A molecular marker linkage map of tetraploid alfalfa (*Medicago sativa* L.). Theor Appl Genet 99:1194–1200

Brouwer DJ, Duke SH, Osborn TC (1998) Comparison of seedlings and cuttings for evaluating winter hardiness in alfalfa. Crop Sci 38:1704–1707

Brouwer DJ, Duke SH, Osborn TC (2000) Mapping genetic factors associated with winter hardiness, fall growth, and freezing injury in tetraploid alfalfa. Crop Sci 40:1387–1396

Brummer EC (1999) Capturing heterosis in forage crop cultivar development. Crop Sci 39:943–954

Brummer EC, Shah MM, Luth D (2000) Reexamining the relationship between fall dormancy and winter hardiness. Crop Sci 40:971–977

Busbice TH (1969) Inbreeding in synthetic varieties. Crop Sci 10:265–269

Busbice TH, Wilsie CP (1965) Fall growth, winter hardiness, recovery after cutting, and witl resistance in F_2 progenies of Vernal × Dupuits alfalfa crosses. Crop Sci 5:429–432

Cao D, Craig BA, Doerge RW (2005) A model selection-based interval-mapping method for autotetraploids. Genetics 169:2371–2382

Champoux MC, Wang G, Sarkarung S, Mackill DJ, O'Toole JC, Huang N, McCouch SR (1995) Locating genes associated with root morphology and drought avoidance in rice via linkage to molecular markers. Theor Appl Genet 90:969–981

Churchill GA, Doerge RW (1994) Empirical threshold values for quantitative trait mapping. Genetics 138:963–971

Daday H, Greenham CC (1960) Genetic studies on cold hardiness in *Medicago sativa* L. J Hered 51:249–255

Demment MW, Teuber LR, Bourque DP, Phillips DA (1986) Changes in forage quality of improved alfalfa populations. Crop Sci 26:1137–1143

Diwan N, Bouton JH, Kochert G, Creagan PB (2000) Mapping of simple sequence repeat (SSR) DNA markers in diploid and tetraploid alfalfa. Theor Appl Genet 101:165–172

Dobrenz AK, Smith SE, Poteet D, Miller WD (1993) Carbohydrates in alfalfa seed developed for salt tolerance during germination. Agron J 85:834–836

Doerge RW, Churchill GA (1996) Permutation tests for multiple loci affecting a quantitative character. Genetics 142:285–294

Dudley JW, Busbice TH, Levings CS (1969) Estimates of genetic variance in Cherokee alfalfa (*Medicago sativa* L.). Crop Sci 9:228–231

Duvick DN (1984) Genetic contribution to yield gains of U.S. hybrid maize, 1930 to 1980. In: Fehr WR (eds) Genetic contributions to yield gains of five major crop plants.Special Publication Number 7. CSSA, Madison, pp 15–47

Dunbier MW, Bingham ET (1975) Maximun heterozygosity in alfalfa: results using haploid-derived autotetraploids. Crop Sci 15:527–531

Echt CS, Kidwell KK, Knapp SJ, Osborn TC, McCoy TJ (1994) Linkage mapping in diploid alfalfa (*Medicago sativa*). Genome 37:61–71

Elden TC, Elgin JH (1987) Recurrent seedling and individual plant selection for potato leafhopper (Homoptera: Cicadellidae) resistance in alfalfa. J Econ Entomol 80:690–695

Groose RW, Talbert LE, Kojis WP, Bingham ET (1989) Progressive heterosis in autotetraploid alfalfa: studies using two types of inbreeds. Crop Sci 29:1173–1177

Heisey RF, Murphy RP (1985) Phenotypic recurrent selection for resistance to *Phytophthora* root rot in two diploid alfalfa populations. Crop Sci 25:693–694

Hill RR, Elgin JH (1981) Effects of number of parents on performance of alfalfa synthetics. Crop Sci 21:298–300

Hill RR, Kalton RR (1976) Current philosophies in breeding for yield. In: Barnes DK (ed) Rep 25th Alfalfa Improve Conf, Ithaca, 13–15 July. USDA-SEA, Peoria/IL, p 51

Hill RR, Shenk JS, Barnes RF (1988) Breeding for yield and quality. In: Hanson AA, Barnes DK, Hill RR (eds) Alfalfa and alfalfa improvement Agron Monogr 29. ASA, CSSA, and SSSA, Madison, pp 809–825

Holland JB, Bingham ET (1994) Genetic improvement for yield and fertility of alfalfa cultivars representing different eras of breeding. Crop Sci 34:953–957

Jones JS, Bingham ET (1995) Inbreeding depression in alfalfa and cross-pollinated crops. Plant Breed Rev 13:209–233

Kaló P, Endre G, Zimányi L, Csanádi G, Kiss GB (2000) Construction of an improved linkage map of diploid alfalfa (*Medicago sativa*). TAG 100:641–657

Kehr WR, Gardner CO (1960) Genetic variability in Ranger alfalfa. Agron J 52:41–44

Kidwell KK, Osborn TC (1992) Simple plant DNA isolation procedures. In: Beckman J, Osborn TC (eds) Plant genomes: methods for genetic and physical mapping. Kluwer, Dordrecht, pp 1–13

Kimbeng CA, Bingham ET (1998a) Population improvement in alfalfa: fertility and S_1 forage yield performance in original and improved populations. Crop Sci 37:1509–1513

Kimbeng CA, Bingham ET (1998b) Population improvement in lucerne (*Medicago sativa* L.): components of inbreeding depression are different in original and improved populations. Austr J Exp Agr 38:831–836

Kimbeng CA, Bingham ET (1999) Population improvement in lucerne (*Medicago sativa* L.): genetic analyses in original and improved populations. Austr J Exp Agr 39:549–554

Maureira IJ, TC Osborn (2005) Molecular markers in genetics and breeding: Improvement of alfalfa (*Medicago sativa L.*). In: Lörz H, Wenzel G (eds) Biotechnology in Agriculture and Forestry. Molecular marker systems in plant breeding and crop improvement, vol 55. Springer-Verlag, Heidelberg, pp 139–154

Maureira IJ, Ortega F, Campos H, Osborn TC (2004) Population structure and combining ability of diverse *Medicago sativa* germplasms. Theor Appl Genet 109:775–782

Miller FR, Kebede Y (1984) Genetic contributions to yield gains in sorghum, 1950 to 1980. In: Fehr WR (eds) Genetic contributions to yield gains of five major crop plants Special Publication Number 7. CSSA, Madison, pp 1–14

Musial JM, Aitken KS, Mackie JM, Irwin JAG (2005) A genetic linkage map in autotetraploid Lucerne adapted to northern Australia, and use of the map to identify DNA markers linked to resistance to *Phytophthora medicaginis*. Austr J Agr Res 56:333–344

Obert DE, Skinner DZ, Stuteville DL (2000) Association of AFLP markers with mildew resistance in autotetraploid alfalfa. Mol Breed 6:287–294

Osborn TC, Brouwer DJ, Kidwell KK, Tavoletti S, Bingham ET (1998) Molecular marker applications to genetics and breeding of alfalfa. In: Brummer EC, Hill NS, Roberts CA (eds) Molecular and cellular technologies for forage improvement. CSSA special publication number 26, Madison, pp 25–31

Pfeiffer TW, Bingham ET (1983) Improvement of fertility and herbage yield by selection within two-allele populations of tetraploid alfalfa. Crop Sci 23:633–636

Quiros CF (1982) Tetrasomic segregation for multiple alleles in alfalfa. Genetics 101:117–127

Riday H, Brummer EC (2002) Forage yield heterosis in alfalfa. Crop Sci 42:716–723

Riday H, Brummer EC (2004) Morphological variation of *Medicago sativa* subsp falcata and their hybrid progeny. Euphytica 138:1–12

Robins JG, Bauchan GR, Brummer EC (2007a) Genetic mapping forage yield, plant height, and regrowth at multiple harvests in tetraploid alfalfa (*Medicago sativa* L.). Crop Sci 47:11–18

Robins JG, Luth D, Campbell TA, Bauchan GR He C, Viands RD, Hansen JL, Brummer EC (2007b) Genetic mapping of biomass production in tetraploid alfalfa. Crop Sci 47:1–10

Rotili P, Gnocchi G, Scotti C, Zannone L (1999) Some aspects of breeding methodology in alfalfa. In: Busbice T, Rotili P, Bingham T (eds) Proceedings of 'The Alfalfa Genome' Conf. (http://www.naaic.org/TAG/TAGpapers/rotili/rotili.html)

Rowe DE, Hill RR (1981) Inter-population improvement procedures for alfalfa. Crop Sci 21:392–397

Salter R, Miller-Garvin JE, Viands DR (1994) Breeding for resistance to alfalfa root caused by *Fusarium* species. Crop Sci 34:1213–1217

SAS Institute (2001) SAS/STAT® Users guide, version 8.02. SAS Inst, Cary

Schwab PM, Barnes DK, Sheaffer CC (1996) The relationship field winter injury and fall growth score for 251 alfalfa cultivars. Crop Sci 36:418–426

Scotti C, Pupilli F, Salvi S, Arcioni S (2000) Variation in vigour and in RFLP-estimated heterozygosity by selfing tetraploid alfalfa: new perspectives for the use of selfing in alfalfa breeding. TAG 101:120–125

Segovia-Lerma A, Murray LW, Townsend MS, Raym IM (2004) Population-based diallel analyses among nine historically recognized alfalfa germplasms. Theor Appl Genet 109:1568–1575

Sills GR, Bridges W, Al-Janabi SM, Sobral B (1995) Genetic analysis of agronomic traits in a cross between sugarcane (*Saccharum officinarum* L.) and its presumed progenitor (*S robustum* Brandes and Jews Ex Grassl). Mol Breed 1:355–363

Smith D (1961) Association of fall growth habit and winter survival in alfalfa. Agron J 41:244–251

Sokal RR, Rohlf FJ (1995) Biometry. Freeman, New York

Sriwatanapongse S, Wilsie CP (1968) Intra- and intervariety crosses of *Medicago sativa* L and *Medicago falcata* L. Crop Sci 8:465–466

Stanford EH (1951) Tetrasomic inheritance in alfalfa. Agron J 43:222–225

Sumberg JE, Murphy RP, Lowe CC (1983) Selection for fiber and protein concentration in a diverse alfalfa population. Crop Sci 23:11–14

Tavoletti S, Veronessi F, Osborn TC (1996) RFLP linkage map of a meiotic mutant based on an F1 population. J Hered 87:167–170

Van Wijk AJP, Reheul D (1991) Achievements in fodder crop breeding in maritime Europe. In: Proceedings of the 16th meeting, fodder crops section, Eucarpia 13. Pudoc, Wageningin, pp 13–18

Waldron LR (1920) First generation crosses between two alfalfa species. J Am Soc Agron 12:133–143

Woodfield DR, Bingham ET (1995) Improvement in two-allele autotetraploid populations of alfalfa explained by accumulation of favorable alleles. Crop Sci 35:988–994

Woodfield DR, Caradus JR (1994) Genetic gain in white clover representing six decades of plant breeding. Crop Sci. 34:1205–1213