Genetic Improvement of Key Agronomic Traits in *Melilotus albus*

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ABSTRACT

Melilotus albus Medik. is a legume species with potential to be used as a forage or conserved fodder in Northwest China. Breeding *M. albus* as a forage crop is focused on simultaneously improving forage yield and reducing coumarin (Cou) content. The goal of the present study was to estimate genotypic variation for several key agronomic traits and to evaluate the impact of single-trait selection and Smith–Hazel-index-based multitrait selection for herbage dry weight (DW), plant height (PH), stem number (SN), and leaf area (LA). A quantitative genetic analysis was conducted on trait measurements from 25 half-sib (HS) families evaluated at Yuzhong and Linze, in China. Significant ($P < 0.05$) genotypic variation among the HS families for all traits was observed. There was also significant ($P < 0.05$) genotype × environment interaction for all traits except for LA. The predicted genetic gain, based on estimated genotypic variance, for single-trait selection varied from 2.3 to 30.3% per selection cycle. Pattern analysis of the HS family × multitrait best linear unbiased prediction matrix provided a graphical summary of the association among the traits, as well as the four HS family groups generated from clustering. Some members within Group 1 could be crossed to generate a breeding population to be used in recurrent selection for *M. albus* cultivar development in Northwest China. The applicability of a Smith–Hazel selection index to increase expression of the traits DW, PH, and SN and reduce LA, indirectly reducing Cou content, was confirmed. This index enabled identification of families for increasing key biomass traits and decreasing Cou production.

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Abbreviations: BLUP, best linear unbiased predictor; Cou, coumarin; DW, herbage dry weight; HS, half-sib; LA, leaf area; LS, leaf-to-stem ratio; PCA, principal component analysis; PH, plant height; REML, restricted maximum likelihood; SD, stem diameter; *w*, weighting coefficients; SH, Smith–Hazel; SN, stem number; SV, spring vigor.

MELILOTUS is a genus of forage legumes native to Eurasia and North Africa (Smith and Gorz, 1965; Stevenson, 1969). The genus is divided into ~19 species. The *Melilotus* genus has adapted to extreme environmental conditions such as cold and drought (Stevenson, 1969) and can grow in soil with moderate salinity where other traditional forage legumes are not productive (Maddaloni, 1986). The high nitrogen fixation rate of *Melilotus* makes the species useful in crop rotation systems (Stickler and Johnson, 1959). Furthermore, members of *Melilotus* are used as ground cover, soil stabilizers, and nectar sources in some countries (Turkington et al., 1978; Allen and Allen, 1981). Certain species of the *Melilotus* genus, such as *Melilotus albus* Medik., have been reported as species with potential for forage production (Rogers et al., 2008). Similar to *M. officinalis* (L.) Lam., *M. albus* is widely spread in Northern China, where it is used as nectar source, ground cover, and a medicinal crop. However, *M. albus* has not been widely used as forage or

conserved fodder due to its high concentration of coumarin (Cou). Coumarin, a plant secondary metabolite derived from the phenylpropanoid biosynthesis pathway, is a precursor dicoumarol content. Dicoumarol is an anticoagulant, and high concentrations in forage or conserved fodder can cause a hemorrhagic condition (Evans and Kearney, 2003; Nair et al., 2010). The development of new *Melilotus* cultivars with low Cou content will be significant to the forage industry. A range of cultivars or breeding lines of *Melilotus* have been released to date, such as Norgold, N28, and N29 (Goplen, 1981; Gorz et al., 1992) for *M. officinalis* and Acuma, Cumino, Polara, and Denta (Smith and Gorz, 1965; Goplen, 1971) for *M. albus*.

Increasing biomass yield is the principal goal for most forage breeding programs. Higher biomass yield improves the economic viability and sustainability of *Melilotus*-based pasture (Perrin et al., 2008). Tysdal and Kiesselbach (1944) pointed out that high-yielding plants were taller and more sparsely leaved. They also had a thicker and woodier stems than low-yielding plants, indicating that it is possible to simultaneously improve forage yield and morphological traits, such as plant height (PH), stem thickness, and stem number (SN). In *M. officinalis*, biomass was correlated with several morphological traits, such as PH, SN, and stem thickness (Luo et al., 2016). A high leaf-to-stem ratio (LS) is also an important agronomic trait that contributes to digestibility and intake in forage (Kephart et al., 1990). Selecting for morphological attributes that contribute to both digestibility and intake of forage biomass would be useful in *Melilotus* breeding directed at improved forage yield.

In plant breeding programs, rate of genetic gain depends on the genetic diversity for a given trait in the breeding population (Hallauer and Miranda, 1981). Available information on genetic variation for different agronomic traits in forage breeding programs will improve the development of breeding strategies to achieve maximum genetic gain (Moll and Stuber, 1974). The development of forage cultivars for the *Melilotus* species will depend on simultaneous selection for increased forage production and yield-related traits while decreasing Cou content. Our breeding program in China is focused on multiple-trait rather than single-trait selection. We have considered applying multitrait selection based on a selection index, which was first proposed by Smith (1936) and further developed by Hazel (1943), the Smith–Hazel (SH) index. Jahufer and Casler (2015) have reported that in switch grass (*Panicum virgatum* L.), combining information on key forage and biofuel production traits into a SH index improved selection efficiency compared with that achieved on selection for forage yield alone. Estimation of genetic variance components for key attributes in plant breeding programs will help breeders determine the efficiency of methods used for genotype evaluation and selection (Hansche et al., 1972). Genetic variation for agronomic traits has been estimated in many important forage legumes and grasses such as alfalfa (*Medicago sativa* L.) (Riday and Brummer, 2007), white clover (*Trifolium repens* L.) (Jahufer et al., 2002), and tall fescue (*Festuca arundinacea* Schreb.) (Piano et al., 2007). There is a lack of information on the magnitude of quantitative genetic variation and genotype × environment interaction effects for key agronomic traits in *M. albus*, which will be important for improving breeding strategies for broad adaptation (Cooper et al., 1993).

The objectives of this experiment were (i) to report a study of agronomic performance in a first-generation (F_1) half-sib (HS) family population of *M. albus* across two contrasting environments, (ii) to estimate genotypic and environmental variation for key agronomic traits, (iii) to develop selection indices for simultaneously increasing biomass yield and decreasing Cou content, and (iv) to identify families with superior agronomic performance and low Cou expression.

MATERIALS AND METHODS
Plant Material
Four elite germplasm accessions (PI 593233, PI 595392, Ames 21248, and LX-05) were identified from a set of 49 *M. albus* accessions that were evaluated for agronomic performance and Cou content at Yuzhong, Gansu Province, China, during 2012 to 2013 (results not presented). Genotypes with superior agronomic performance representing each of the four germplasm accessions were then polycrossed under isolation using honey bees (*Apis mellifera* L.), to produce a breeding population to be used for cultivar development. The F_1 HS families were produced by hand harvesting each of the plants in the polycross individually. Twenty-five F_1 HS families were randomly sampled for this study.

Field Trials
The 25 HS families were established at two locations: Yuzhong (35°89′ N, 104°09′ E) and Linze (39°15′ N, 100°02′ E) in Gansu Province, China. These two locations represented the Loess Plateau region and Hexi Corridor, respectively. The climatic conditions were medium temperate semiarid at Yuzhong and typical desert environment (characterized by arid conditions) at Linze (Su et al., 2007; Hu et al., 2012; Bai et al., 2014; Li et al., 2014). The average annual evaporation and precipitation (1980–2010) were 1377 and 372 mm at Yuzhong and were 1997 mm and 113 mm at Linze, respectively. The total monthly rainfall and mean monthly maximum and minimum temperatures during the trial period (May 2014 to August 2015) at the two locations (Yuzhong and Linze) are presented in Fig. 1. The soil types of the two locations were loessal soil and meadow soil, respectively. The salinity content was 3.83 g kg⁻¹ at Yuzhong and 10.21 g kg⁻¹ at Linze. Initial soil conditions (0–30 cm) at trial establishment at Yuzhong and Linze were as follow: pH of 7.0 and 7.5, total P of 0.75 and 0.71 g kg⁻¹, and total N of 0.76 and 0.80 g kg⁻¹, respectively.

At each location, the experimental plot layout was a randomized complete block design containing three replicates.
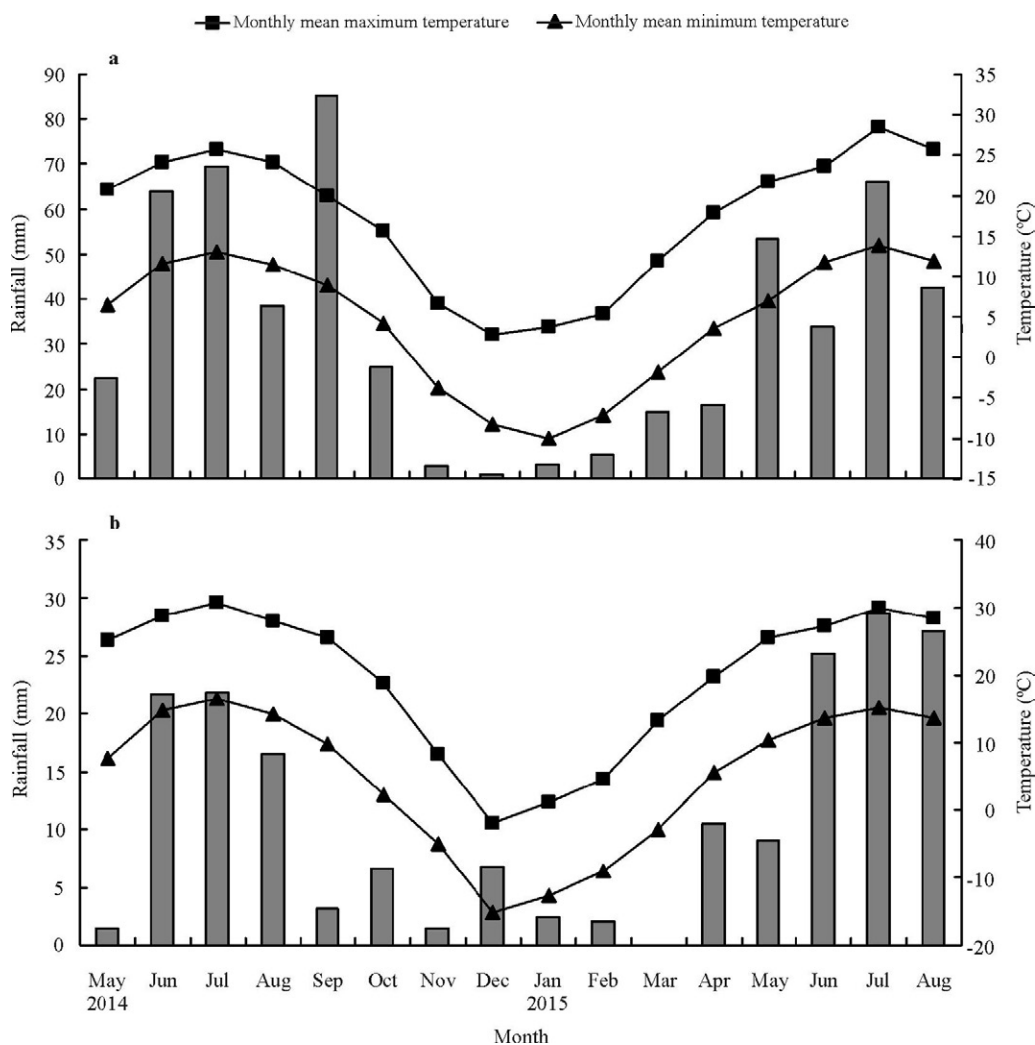
Fig. 1. Total monthly rainfall (mm) and mean monthly maximum and minimum temperatures (°C) during the trial period at (a) Yuzhong and (b) Linze, respectively.

Each replicate consisted of the 25 HS families, two check cultivars and four parental germplasm accessions. Each entry was planted in a 0.8-m × 3-m experimental plot. Within each plot, there was a spacing of 60 cm between plant rows and 30 cm within rows. The origins of these entries are presented in Table 1. The two trials were sown during 15 to 18 June 2014. Within each plot, hole-seeding was used, in which one seed per clump was planted at a depth of 1 to 2 cm. Each plot was fertilized with 150 kg $(NH_4)_2HPO_4$ ha^{-1} after seeding.

Measurements

All traits were measured in 2015 (the second year of *M. albus* growth). Visual scoring for spring vigor (SV) was based on a scale of 1 to 5 (1 = high, 3 = middle, 5 = low). The SV was measured 30 d after the plants turned green. The forage–yield-related morphological traits (stem diameter [SD, cm], SN, PH [cm], and leaf area [LA, cm^2]) were measured at the flowering stage (50% of the plants had open flowers) from three individuals per replicate. Leaf area was measured from three central leaflets of the most fully expand leaves per plant using a flatbed scanner (EPSON GT-15000) and a WinSEEDLE 2011 image analysis system (Regent Instruments). Herbage dry weight (DW, g $plant^{-1}$) for each HS family was based on a random sample of three plants taken per replicate. The random plants were harvested individually for DW after measuring morphological traits. At harvest, three random plants were taken from

each replicate. Each plant was cut 3 cm above the soil surface. The plants were placed in paper bags and dried at room temperature (~20–25°C) until constant weight. After measuring DW, the dried samples were hand separated into leaf blade and stem (including the inflorescence and leaf sheath) components and weighed to determine LS. Three subsamples from each replicate at Yuzhong were combined as a replicate and ground in a mill to pass through a 1-mm screen for further Cou measurement. Coumarin (% of dry matter) was quantified using high-performance liquid chromatography (Agilent 1100 series) with a mobile phase of methanol–water (65:35) through an Agilent-XDB C18 column (Zhu and Fan, 2008).

ANOVA

The data were analyzed within and across the two locations: Yuzhong and Linze. The analysis across locations was conducted

Table 1. Origin of the *M. albus* germplasm accessions and check cultivars.

Accession no.	Source of seed	Latitude and longitude
PI 593233	Wisconsin, USA	43°47′ N, 88°47′ W
PI 595392	Oregon, USA	43°48′ N, 120°33′ W
Ames 21248	Douro Litoral, Portugal	24°46′ N, 8°59′ E
LX-05	Gansu, China	35°89′ N, 104°09′ E
PI 297086	Nebraska, USA	Check cultivar
LX-01	Gansu, China	Check cultivar

(i) on only the 25 first generation HS families to estimate genotypic variation, and (ii) using all entries in the trial that consisted of the 25 HS families, the four parental germplasm accessions, and two check cultivars, which enabled comparison of progeny, parents, and the commercial material. The analysis was conducted using the variance component analysis procedure, residual maximum likelihood (REML) option, in GenStat 7.1 (GenStat, 2003). A mixed linear model was used for the analyses across the two locations using the REML algorithm.

The linear model used in the analysis was

$$Y_{ijk} = M + g_i + l_j + r_{jk} + (gl)_{ij} + \varepsilon_{ijk}$$

where Y_{ijk} is the value of an attribute measured from HS family i in replicate k in location j, and $i = 1,..., n_g$, $j = 1,..., n_l$, and $k = 1,...,n_r$; M is the overall mean; g_i is the random genotypic effect of HS family i, $N(0,\sigma^2_g)$, where σ^2_g is the genotypic variance; l_j is the fixed effect of location j, $N(0, \sigma^2_l)$, where σ^2_l is the location variance; r_{jk} is the random effect of replicate k within location j, $N(0, \sigma^2_b)$, where σ^2_b is the block variance; $(gl)_{ij}$ is the effect between HS family i and environment j, $N(0, \sigma^2_{gl})$, where σ^2_{gl} is the genotype × location interaction variance; and ε_{ijk} is the residual effect for HS family i in replicate k in location j, $N(0, \sigma^2_\varepsilon)$, where σ^2_ε is the error variance.

The mixed-model analysis generated HS family means based on best linear unbiased predictors (BLUP) (White and Hodge, 1989). These BLUP values were used to construct a HS family × trait mean matrix adjusted for HS family × location interaction effects.

Genotypic Variation
Variation among HS families generated from a population that has gone through at least two cycles of random mating is an estimated 0.25 additive variation of the random mating population they represent (Falconer, 1989). In our study, the 25 HS families were a result of the first random mating of selected germplasm and therefore represented only the first generation. Therefore, we do not refer to the variation estimated among the 25 HS families as 0.25 additive variation, but as genotypic variation, due to a possible combination of additive and nonadditive effects.

Pattern Analysis
Pattern analysis was conducted (i) to provide a graphical summary of the performance of the 25 HS families, four parental germplasm accessions, and two check cultivars of *M. albus* based on the genotype × trait BLUP adjusted mean matrix generated from variance component analysis across the two locations (Yuzhong and Linze), and (ii) to investigate any changes in type (positive or negative) and magnitude of the association among the seven traits across Yuzhong and Linze. Pattern analysis consisted of a combination of cluster and principal component analysis (PCA) (Gabriel, 1971; Kroonenberg, 1994; Watson et al., 1995). To identify the optimum level of truncation for the resulting hierarchy from cluster analysis, the increase in the sum of squares among accession groups was monitored as the number of groups increased. The group level selected was determined by the point where the percentage of accession sum of squares among groups did not improve substantially as the number of groups increased (DeLacy, 1981).

Phenotypic and Genotypic Correlation
Phenotypic correlation (r_p) analysis was performed using GenStat 7.1 (GenStat, 2003). The multivariate ANOVA procedure within GenStat 7.1 enabled estimation sums of cross-products using the multisite trait data from the 25 HS families. Mean cross products were then calculated and resolved to estimate genotypic covariance components. The genotypic covariance components between traits were used together with their individual genetic variance estimates, from REML analysis, to determine genotypic correlation coefficients (r_g) according to Falconer (1989).

Expected Genetic Gain
Expected genetic gain (ΔG) was estimated for all individual traits with significant additive genetic variance among the 25 HS families. The ΔG was estimated using the equation according to Casler and Brummer (2008) for HS family selection in obligate outcrossing forage crops;

$$\Delta G = k_f c \frac{\frac{1}{4}\sigma^2_A}{\sigma_{PF}}$$

where k_f is the standardized selection differential among families, c is the parental control factor, σ^2_A is the additive genetic variance (in this study taken as genotypic variance), and σ_{PF} is the phenotypic standard deviation among families. For HS family selection, $c = 0.5$, as selection is on female gametes only (Falconer, 1989). This study assumed a selection pressure of 20% ($k_f = 1.4$).

Smith-Hazel Index
In this study, the SH index (Smith, 1936; Hazel, 1943) was used to develop index coefficients that would identify superior HS families associated with increased DW, PH, and SN and decreased LA. The SH index:

$$\mathbf{b} = \mathbf{P}^{-1}\mathbf{Aw}$$

where **P** and **A** are phenotypic and additive genetic (in this study taken as genotypic) covariance matrices, respectively, and **b** and **w** are vectors of index coefficients and economic weightings, respectively.

The SH index constructed for three traits with increasing DW (x) and PH (y) and decreasing Cou ($-z$):

$$\begin{pmatrix} b_x \\ b_y \\ b_z \end{pmatrix} = \begin{pmatrix} V_{P_x} & \text{Cov}_{P_{xy}} & \text{Cov}_{P_{xz}} \\ \text{Cov}_{P_{xy}} & V_{P_y} & \text{Cov}_{P_{yz}} \\ \text{Cov}_{P_{xz}} & \text{Cov}_{P_{yz}} & V_{Pz} \end{pmatrix}^{-1}$$

$$\times \begin{pmatrix} V_{A_x} & \text{Cov}_{A_{xy}} & \text{Cov}_{A_{xz}} \\ \text{Cov}_{A_{xy}} & V_{A_y} & \text{Cov}_{A_{yz}} \\ \text{Cov}_{A_{xz}} & \text{Cov}_{A_{yz}} & V_{Az} \end{pmatrix} \begin{pmatrix} w_x \\ w_y \\ -w_z \end{pmatrix}$$

where b is the resulting index coefficient, V_P is the phenotypic variance, Cov_P is the phenotypic covariance, V_A is the additive variance (in this study taken as genotypic variance), Cov_A is the genetic covariance, and w is the optimum weighting coefficient value. Several simulations with varying w combinations were

conducted to identify the appropriate set that would enable increasing DW, PH, and SN and decreasing LA.

The genetic worth (*I*) of an individual HS family based on traits *x*, *y*, and *z* was estimated according to:

$$I = b_x \overline{HS}_x + b_y \overline{HS}_y + b_z \overline{HS}_z$$

where *b* is the index coefficient for the traits *x*, *y*, and *z*; and HS is the individual HS family BLUP adjusted mean based on analysis across the two locations (Yuzhong and Linze) for the traits *x*, *y*, and *z*.

The optimum set of *w* associated with index coefficients (*b*) that would generate HS family indices (*I*), resulting in achieving the desired predicted genetic gain for each of the four traits at a specific selection pressure, was estimated according to Van Vleck et al. (1987):

$$\Delta G_Y = k_f c r_{IY} \sigma_{PY}$$

where ΔG_Y predicted genetic gain for individual trait *Y* in the index, k_f was among-HS-family selection pressure, *c* was parental control, r_{IY} was the correlation between the calculated SH indices *I* for the individual HS families and their BLUP values for trait *Y*, and σ_{PY} was the among-HS-family phenotypic standard deviation of the BLUP values for trait *Y*.

RESULTS
Genotypic Variance Components of Plant Attributes of *M. albus*

The trait means and ranges generated from this study indicated a wide range of phenotypic variation for plant dry matter, the morphological traits measured, and Cou content in *M. albus*. The genotypic variances estimated among the 25 HS families for the different traits based on within-location REML analysis were significant (*P* < 0.05) (Table 2, 3, and 4). Restricted maximum likelihood analysis for mean trait expression across the two locations also showed significant (*P* < 0.05) genotypic variation (σ^2_g) among the 25 HS families. There was significant (*P* < 0.05) genotype × location interaction (σ^2_{gl}) for all traits expect for LA (Table 4).

Pattern Analysis

The biplot generated from PCA of the 25 HS families, the two check cultivars, and the four parental germplasm accessions of *M. albus* based on the six key traits (DW, SD, SN, PH, LA and Cou) graphically summarized the HS family BLUP adjusted mean matrix (Fig. 2). The first principal component explained 48% of the total trait variation, and the second principal component explained 24%. The correlation structure of the traits is indicated by the directional vectors in the biplot. The traits PH, SD, and SN showed a strong positive association with DW. The trait Cou showed a strong positive association with LA.

Clustering of the 25 *M. albus* HS families, together with the two check cultivars and four parental germplasm accessions, was truncated at the four-group level. Groups 3 and 4, the largest groups, each contained 10 members, followed by Groups 1 and 2, which contained eight and three

Table 2. Trait average, maximum, minimum, least significant differences (LSD$_{0.05}$), genotypic (σ^2_g) and experimental error (σ^2_ε) variance components, and associated standard errors (±SE), estimated from the 25 *M. albus* half-sib families, evaluated at Yuzhong.

Statistic	Trait†						
	DW	LS	PH	SD	SN	SV	LA
	g plant⁻¹		——— cm ———				cm²
Avg.	124	1.03	168	2.0	7.8	2.2	7.6
Max	192	1.29	203	3.1	9.9	4.7	8.3
Min	26	0.80	129	1.2	6.3	0.8	6.8
LSD$_{0.05}$	22	0.14	14	0.2	1.3	0.7	0.5
σ^2_g	1614 ± 476	0.023 ± 0.007	236 ± 74	0.31 ± 0.09	0.67 ± 0.28	0.61 ± 0.19	0.28 ± 0.09
σ^2_ε	327 ± 30	0.021 ± 0.002	175 ± 16	0.04 ± 0.01	2.71 ± 0.25	0.18 ± 0.03	0.10 ± 0.02

† DW, herbage dry weight; LS, leaf-to-stem ratio; PH, plant height; SD, stem diameter; SN, stem number; SV, spring vigor; LA, leaf area.

Table 3. Trait average, maximum, minimum, least significant differences (LSD$_{0.05}$), genotypic (σ^2_g) and experimental error (σ^2_ε) variance components and associated standard errors (±SE), estimated from the 25 *M. albus* half-sib families, evaluated at Linze.

Statistic	Trait†						
	DW	LS	PH	SD	SN	SV	LA
	g plant⁻¹		——— cm ———				cm²
Avg.	135	0.97	164	1.7	8.5	2.0	7.8
Max	194	1.26	207	2.3	10.9	3.5	8.5
Min	31	0.87	111	0.8	7.1	0.1	6.8
LSD$_{0.05}$	23	0.12	15	0.3	1.5	1.0	0.7
σ^2_g	2260 ± 663	0.009 ± 0.003	504 ± 152	0.18 ± 0.05	1.03 ± 0.40	0.71 ± 0.22	0.32 ± 0.10
σ^2_ε	321 ± 29	0.018 ± 0.002	191 ± 17	0.06 ± 0.01	3.28 ± 0.30	0.17 ± 0.03	0.08 ± 0.01

† DW, herbage dry weight; LS, leaf-to-stem ratio; PH, plant height; SD, stem diameter; SN, stem number; SV, spring vigor; LA, leaf area.

Table 4. Trait average, maximum, minimum, least significant differences (LSD$_{0.05}$), phenotypic (σ^2_P), and genotypic (σ^2_g), genotype-by-location interaction (σ^2_{gl}), and experimental error (σ^2_ε) variance components, and associated standard errors (±SE), estimated from the 25 *M. albus* half-sib families, evaluated across two locations, Yuzhong and Linze.

Statistic	Trait†							
	DW	LS	PH	SD	SN	SV	LA	Cou‡
	g plant⁻¹		——— cm ———				cm²	% dry matter
Avg.	130	1.00	166	1.8	6.0	2.4	7.7	0.498
Max	188	1.27	206	2.6	9.0	3.7	8.4	1.111
Min	48	0.83	134	1.1	4.2	1.7	6.7	0.208
LSD$_{0.05}$	17	0.10	11	0.2	0.9	0.8	0.4	0.087
σ^2_g	1006 ± 434	0.009 ± 0.004	194 ± 85	0.11 ± 0.05	0.82 ± 0.30	0.34 ± 0.01	0.27 ± 0.07	0.032 ± 0.009
σ^2_{gl}	920 ± 268	0.004 ± 0.003	163 ± 55	0.13 ± 0.04	0.24 ± 0.13	0.55 ± 0.16	NS§	–
σ^2_ε	137 ± 20	0.016 ± 0.002	96 ± 14	0.03 ± 0.01	0.64 ± 0.09	0.20 ± 0.03	0.09 ± 0.01	0.001 ± 0.0003
σ^2_P	2063	0.03	454	0.27	1.70	0.85	0.39	0.03
$\sqrt{\sigma^2_P}$	45	0.17	21	0.52	1.31	0.92	0.62	0.18

† DW, herbage dry weight; LS, leaf-to-stem ratio; PH, plant height; SD, stem diameter; SN, stem number; SV, spring vigor; LA, leaf area; Cou, coumarin.

‡ This trait was only evaluated in one location (Yuzhong).

§ NS, not significant ($P < 0.05$).

members, respectively (Fig. 2, Table 5). The check cultivars MAC1 and MAC2 in our study were both in Group 4. The parental germplasm accessions MAP1, MAP2, MAP3, and MAP4 were distributed across all four groups. The means of all the key agronomic traits for the four groups are shown in Table 5. The members in Group 1 had high DW and low Cou expression, and those in Group 2 had high DW, PH, SD, and SN and intermediate Cou expression. The members from Group 4 showed characteristics of a small plant type, and also low Cou content. The members in Group 3 showed the highest expression for Cou.
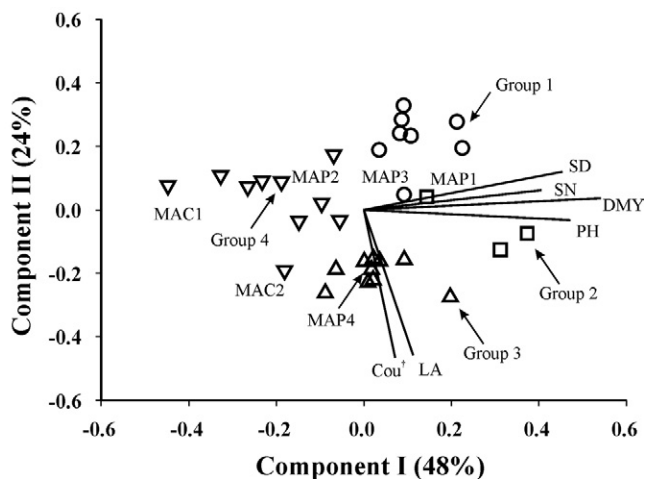

Fig. 2. Biplot generated using standardized best linear unbiased predictor (BLUP) values for six key agronomic traits measured from: the 25 half-sib families, the two check cultivars, and the four parental germplasm accessions of *M. albus*, evaluated across two locations, Yuzhong and Linze. The different symbols indicate Groups 1 to 4 generated from cluster analysis. The vectors represent the traits: SD, stem diameter (cm); SN, stem number; PH, plant height (cm); LA, leaf area (cm²); DW, herbage dry weight (g plant⁻¹); Cou, coumarin (% of dry matter). The four parental germplasm accessions: MAP1 to MAP4. Check cultivars: MAC1 and MAC2. † Indicates that this trait was only evaluated at one location (Yuzhong).

Phenotypic and Genotypic Correlation

The genotypic and phenotypic correlation coefficients are presented in Table 6. These coefficients range from strong to weak positive or negative pairwise associations between the eight traits. Of special interest are the phenotypic and genotypic correlations between DW and the other traits. There were strong positive phenotypic correlations between DW and the traits PH, SD, and SN and strong negative phenotypic correlations with LS and SV. There was a strong correlation between Cou and LA, but no correlation between Cou and any other trait. The estimated genotypic correlation coefficients for all traits showed similar types of pairwise association, as indicated by the phenotypic correlations (Table 6).

Single-Trait Selection and Smith–Hazel-Index-Based Selection

Selection on an individual-trait basis at 20% selection pressure resulted in a range of predicted genetic gains (%ΔG) depending on the trait. The predicted genetic gain, based on estimated genotypic variance, for single-trait selection varied from a low 2.3% for SV to a high 30.3% for Cou, per cycle of selection. There was a predicted 11.6% increase per cycle of selection for DW (Table 7).

The optimum set of weighting coefficients (*w*) derived after several iterations of SH index simulation to increase DW yield and simultaneously increase PH and SN, and to minimize response to selection of LA, is presented in Table 8. These were 0.15, 0.38, 0.30, and −0.90 for DW, PH, SN, and LA, respectively. The resulting expected genetic gain per cycle of multitrait selection, based on the SH index, for the individual traits DW, PH, SN, and LA were 10.9, 3.5, 6.4, and 1.0%, respectively.

Table 5. Trait means for each of the four half-sib family groups generated from pattern analysis.

Group	No. in group	Trait†					
		DW	PH	SD	SN	LA	Cou
		g plant^{-1}	——— cm ———			cm^2	% dry matter
1	8	184	187	2.1	7.1	7.2	0.25
2	3	201	197	2.4	7.8	8.3	0.40
3	10	156	180	1.7	6.6	8.2	0.66
4	10	104	158	1.4	6.1	7.5	0.35

† DW, herbage dry weight; PH, plant height; SD, stem diameter; SN, stem number; LA, leaf area; Cou, coumarin.

Table 6. Genotypic (r_g, lower triangle) and phenotypic (r_p, upper triangle) correlation coefficients between traits based on the 25 *M. albus* half-sib families, the four parental germplasm accessions, and the two check cultivars, evaluated across two locations, Yuzhong and Linze.

Trait	Trait†							
	PH	DW	LS	SD	SN	SV	LA	Cou‡
	cm	g plant^{-1}		cm			cm^2	% dry matter
PH		0.66**	−0.13	0.41**	0.21*	−0.59**	0.12	−0.01
DW	0.638		−0.23*	0.68**	0.51**	−0.82**	0.08	−0.06
LS	−0.301	−0.491		−0.25*	−0.05	0.22*	0.10	0.02
SD	0.734	0.937	−0.577		0.30*	−0.52**	−0.08	−0.12
SN	0.245	0.761	−0.222	0.555		−0.43**	0.13	−0.01
SV	−0.507	−0.947	0.415	−0.802	−0.924		−0.11	0.03
LA	0.222	0.130	0.199	−0.122	0.221	−0.235		0.62**
Cou‡	−0.005	−0.054	0.059	−0.144	−0.014	0.021	0.738	

*,** Significant at the 0.05 and 0.01 probability levels, respectively.

† PH, plant height; DW, herbage dry weight; LS, leaf-to-stem ratio; SD, stem diameter; SN, stem number; SV, spring vigor; LA, leaf area; Cou, coumarin.

‡ This trait was only evaluated in one location (Yuzhong).

DISCUSSION

There is good evidence that plant breeding has successfully improved populations whenever there is genetic variation within germplasm pools and selection has focused on the right traits measured in the appropriate environments (Cooper et al., 2014). Here, we evaluated HS families derived from random mating of genotypes from elite germplasm accessions and estimated quantitative genetic parameters for key traits. These are important steps towards identifying an efficient breeding method to maximize genetic gain per cycle of selection. Previous studies

Table 7. Predicted genetic gain (ΔG) per selection cycle in absolute values and percentage (%ΔG) in response to direct single-trait selection, based on the performance of the 25 *M. albus* half-sib families evaluated across two locations, Yuzhong and Linze. Selection intensity = 20% (k = 1.4).

Traits†	ΔG	%ΔG change
DW (g plant^{-1})	18.25	11.6
LS	0.05	5.7
PH (cm)	7.95	4.4
SD (cm)	0.18	10.0
SN	0.56	8.2
SV	0.04	2.3
LA (cm^2)	0.38	4.9
Cou‡ (% of dry matter)	−0.15	−30.3

† DW, herbage dry weight; LS, leaf-to-stem ratio; PH, plant height; SD, stem diameter; SN, stem number; SV, spring vigor; LA, leaf area; Cou, coumarin.

‡ This trait was only evaluated in one location (Yuzhong).

on genotypic variation have focused on genetic diversity (Di et al., 2014; Wu et al., 2016) and the phylogenic relationships (Di et al., 2015) within the *Melilotus* genus by using molecular markers and DNA sequencing. The presence of genotype × environment interactions complicates selection of material for broad adaptation due to variable relative performances across environments (Comstock and Moll, 1963; Cooper and Byth, 1996). Quantifying the magnitude and understanding the causes of genotype × environment interaction can be helpful when planning breeding strategies (Milligan et al., 1990; Basford and Cooper, 1998). Caradus (1993) reported that a range of traits in white clover, especially yield-related traits, were sensitive to genotype × environment interactions. Similar results were also reported by Jahufer et al. (2009) from a white clover plot trial evaluating a range of experimental synthetics in Australia. In our study, there was significant genotype × environment interaction for most traits, indicating the importance of multisite evaluation. It is essential that *M. albus* breeding programs are focused on broad adaptation and include multisite evaluation across the target population of environments. The positive and significant phenotypic association of DW with traits PH, SD, and SN predicts a positive correlated response in all these traits when selection is based on any one of them. Our study indicated negative phenotypic and genotypic correlation between DW and LS. The LS is used as an

Table 8. The final sets of weighting coefficients used in the construction of Smith–Hazel selection indices for four key traits, predicted genetic gain (ΔG) for individual trait selection, and individual trait ΔG resulting from index selection, using the 25 *M. albus* half-sib families evaluated across two locations. The selection intensity = 20% (*k* = 1.4).

Selection traits†	Final set of weightings used in index	ΔG from individual trait selection absolute values	ΔG from index selection absolute values
DW (g plant⁻¹)	0.15	18.25 (11.6%)	17.07 (10.9%)
PH (cm)	0.38	7.95 (4.4%)	6.32 (3.5%)
SN	0.30	0.56 (8.2%)	0.44 (6.4%)
LA (cm²)	−0.90	−0.38 (4.9%)	−0.08 (1.0%)

† DW, herbage dry weight; PH, plant height; SN, stem number; LA, leaf area.

indicator of digestibility and intake in forage (Kephart et al., 1990). This result implies a tradeoff between herbage yield and quality, and similar results were reported from studies on alfalfa (Julier et al., 2000) and yellow sweetclover (*Melilotus officinalis* L.) (Luo et al., 2016).

The basic concept of using the SH index is to define the genetic worth of an individual on the basis of a linear function of genetic values of multiple traits, each weighted to a predetermined relative economic value (Baker, 1974). The advantage of this index is that it combines information on heritability, correlations among traits, and also economic importance if available (Cotterill and Dean, 1990). In our study, the objective was to increase the expression of key biomass yield components and decrease Cou, using a selection-generated index based on the SH equation. The index generated, based on the set of *w* after several iterations of simulation using a 20% selection intensity, resulted in predicting successful increases of DW, PH, and SN (all traits associated with plant biomass) and a decrease of LA, which has a strong positive phenotypic and genotypic correlation with Cou. In comparison with individual-trait, HS-family-based selection, the index-based genetic gains were lower for DW, PH, and SN. However, index-based selection enabled identification of families with an appropriate combination of higher DW, PH, and SN and low Cou expression, significant for *M. albus* cultivar development in China. The SH index has been successfully used in the breeding of a number of crop species, such as maize (*Zea mays* L.) (Bänziger and Lafitte, 1997), barley (*Hordeum vulgare* L.) (Eshghi et al., 2011), wheat (*Triticum aestivum* L.) (Gebre-Mariam and Larter, 2006), and several tree species (Cotterill and Dean, 1990). However, the reported application of the SH index in forage grass breeding has been limited to a few examples. Jahufer and Casler (2015) reported that combining information on forage yield and quality in a SH index improved selection efficiency in a switchgrass breeding program to increase biomass and ethanol while reducing Klason lignin to develop cultivars for a fermentation platform.

Crossing of genetically divergent populations could result in progeny outperforming the parental populations (Brummer, 1999). Busbice et al. (1974) reported that interpopulation crosses of extremely diverse alfalfa accessions resulted progeny yields of up to nearly 200% of the higher parent value. Our study revealed a clear pattern whereby elite *M. albus* germplasm accessions generally gave rise to superior progeny in the first-generation HS families. We cannot rule out the possibility that hybrid vigor in this species may confound assessment of ecotype germplasm for additive sources of variation in first-generation crosses. However, our study provides a preliminary assessment of the genetic parameters for designing a breeding strategy for the second-generation (F₂) HS families currently being developed. Results from our work also demonstrated the potential merit of using index selection in *M. albus* breeding. Finally, if heterosis exists, it may offer another potential method to improve *M. albus* via semihybrid cultivars.

CONCLUSION

There was significant (*P* < 0.05) genotypic variation for all the traits examined in the present study. Genotype× environment interaction was significant (*P* < 0.05) for the traits SV, DW, and morphological traits SD, SN, PH, and LS across the two locations, Yuzhong and Linze. The estimates of genotypic variation indicated the potential genetic variation available for key agronomic traits of *M. albus*. The breeding population developed by polycrossing the HS families within Group 1 generated from pattern analysis will provide a useful breeding pool for *M. albus* cultivar development in China. The SH Index constructed to increase DW and reduce Cou will enhance selection of elite HS families for the development of new high-biomass-yielding, low-Cou-expressing *M. albus* cultivars.

Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

Kai Luo, M.Z.Z. Jahufer, Jiyu Zhang, and Yanrong Wang conceived the topic. Kai Luo, Hong Zhao, Rui Zhang, Fan Wu, and Zhuanzhuan Yan performed the experiments. Kai Luo and M.Z.Z. Jahufer analyzed all statistical data. Kai Luo wrote the manuscript. All authors revised the manuscript.

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