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Review

Applied Genetics and Genomics in Alfalfa Breeding

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Abstract: Alfalfa (*Medicago sativa* L.), a perennial and outcrossing species, is a widely planted forage legume for hay, pasture and silage throughout the world. Currently, alfalfa breeding relies on recurrent phenotypic selection, but alternatives incorporating molecular marker assisted breeding could enhance genetic gain per unit time and per unit cost, and accelerate alfalfa improvement. Many major quantitative trait loci (QTL) related to agronomic traits have been identified by family-based QTL mapping, but in relatively large genomic regions. Candidate genes elucidated from model species have helped to identify some potential causal loci in alfalfa mapping and breeding population for specific traits. Recently, high throughput sequencing technologies, coupled with advanced bioinformatics tools, have been used to identify large numbers of single nucleotide polymorphisms (SNP) in alfalfa, which are being developed into markers. These markers will facilitate fine mapping of quantitative traits and genome wide association mapping of agronomic traits and further advanced breeding strategies for alfalfa, such as marker-assisted selection and genomic selection. Based on ideas from the literature, we suggest several ways to improve selection in alfalfa including (1) diversity selection and paternity testing, (2) introgression of QTL and (3) genomic selection.

Keywords: alfalfa; breeding; phenotypic selection; QTL mapping; association mapping; marker-assisted selection; genomic selection

1. Introduction

Alfalfa, (*Medicago sativa* L.) a perennial and outcrossing species, originated in and around Transcaucasia and possibly also in central Asia; for a comprehensive review of alfalfa's origins, see Small [1] and Russelle [2]. Today, alfalfa is one of the most widely planted forage legumes for hay, pasture and silage in the world because of its highly nutritious forage and broad adaptability. Alfalfa also has significant benefits for sustainable cropping systems because its deep root system and perennial nature limit soil erosion and improve soil tilth. As a legume, alfalfa forms a symbiotic association with the soil bacterium *Sinorhizobium meliloti*, which fixes atmospheric nitrogen (N), providing N for the plant and increasing soil N fertility for subsequent crops in a rotation. Alfalfa has a genome size of about 800–1000 Mbp [3], has a base chromosome number of $x = 8$, and exists at two ploidy levels (diploid, $2n = 2x = 16$, and tetrasomic tetraploid, $2n = 4x = 32$).

In this paper, we discuss alfalfa germplasm diversity and explain the common breeding methods currently used to develop alfalfa cultivars. We then review the advances in genetic mapping and quantitative trait loci (QTL) detection that have been made in alfalfa and present our view of how genomic tools will impact future alfalfa cultivar development programs. We focus on the use of molecular markers to assess genetic diversity and population structure, to augment selection programs, and to implement genomic selection (GS). We also discuss some potential challenges and considerations when applying these tools to alfalfa breeding programs.

2. Alfalfa Origins and Genetic Diversity

Two primary subspecies exist at each ploidy level, subsp. *falcata* with yellow flowers and falcate pods, and subsp. *sativa* with purple flowers and coiled pods (named subsp. *caerulea* at the diploid level). Natural hybrids between the two subspecies have been found at both ploidy levels [4]. Inter-subspecies hybrids show normal meiosis [5] and equivalent or superior fertility compared to intra-subspecies hybrids [6]. Describing the full complexity of the *M. sativa* taxon is outside the purview of this paper; the interested reader is directed to the monograph by Small [1], which comprehensively discusses this and other *Medicago* species.

Alfalfa domestication probably occurred in multiple locations and the dates of domestication are unclear [1]. However, alfalfa was mentioned in Babylonian texts in 700 BC [7] suggesting that it was cultivated by that time. From the center(s) of origin, alfalfa spread throughout much of Europe, North Africa, the Middle East and Central and Northern Asia. Alfalfa germplasm was introduced into North and South America, beginning in the 16th century, and into Australia in the 1800s [8]. Today, exchange of germplasm between all alfalfa growing regions continues, both through germplasm collections of wild or feral populations and through commercial sales or acquisition of elite breeding material by commercial breeding companies.

Many early experiments using genetic markers to assess alfalfa genetic diversity have been previously reviewed [9] and here we concentrate on subsequent experiments. An extensive examination of wild diploid germplasm using simple sequence repeat (SSR) markers showed that *falcata* and *caerulea* accessions are clearly distinct, with hybrids falling in between the separate

parental groups [10,11]. *Falcata* and *sativa* are similarly distinct, based on numerous experiments conducted on tetraploid populations [9,12,13].

Tetraploids have apparently arisen through recurrent polyploidization via unreduced gametes. Examination of mitochondrial, chloroplast, and nuclear genes and DNA markers clearly show that *sativa* is the tetraploid form of *caerulea* [13–15]. Although the data are generally consistent with recurrent polyploidization and interploidy gene flow, nuclear gene sequences also support the existence of a bottleneck in the domestication of at least some alfalfa populations [15]. Tetraploid *falcata* origins appear to be more complex, with some evidence of introgression from *M. prostrata* [13]. Mixing between cultivated and wild populations has been documented [16], but some wild populations appear to have remained distinct despite cultivars being grown in the vicinity [17].

The extant DNA sequence variation in tetraploid alfalfa populations is large by virtually any standard. As much as 99% of marker DNA variation can be found within populations [18]. The level of genetic variation among a series of related cultivars derived from the same breeding pool suggested relatively little loss of alleles through the breeding process [19]. Recently, we have analyzed transcriptomes of 27 genotypes, including 16 elite genotypes from four US alfalfa breeding companies and five from uncultivated populations (two diploid *falcata*, two diploid *caerulea* and one tetraploid *falcata* genotype) (Li *et al*., in preparation). Over 90% of single nucleotide polymorphisms (SNP) identified among the five non-cultivated genotypes were still in existence among the 16 elite commercial clones. While our sample is not large, the fact that so many SNP identified from a small sample of unimproved germplasm were still present in cultivated germplasm suggests that the domestication and selection bottleneck was not large.

The diversity experiments to date can be summarized as follows. Alfalfa spread naturally across Eurasia and North Africa in historical times, probably facilitated by armies carrying hay for draft animals [1]. Domestication likely occurred multiple times at multiple locations from different initial diploid germplasm. Subsequently, over the past several centuries, explorers and seedsmen have transferred alfalfa germplasm around the world. As a consequence, much of the genetic variation found in wild populations persists even in elite breeding material (although allele frequencies have presumably changed), and the amount of DNA sequence variation present within any given population (whether a cultivar or germplasm) remains very high.

3. Alfalfa Breeding

3.1. Breeding Goals and Traits of Interest

Many breeding goals for alfalfa are similar to those in other crops—increasing yield, enhancing nutritive value and improving tolerance to abiotic and biotic stresses are all important. Many of these agronomic traits, including yield, nutritive value and persistence are quantitatively inherited but at least some disease and pest resistances are likely under simple genetic control. Alfalfa breeders have been mainly focused on improving disease and insect resistance, forage nutritive value and, in colder regions, winter hardiness or frost tolerance to reduce stand loss. Since at least the 1940's, diverse germplasms have been combined to enable selection for resistance to multiple diseases and/or

pests [8]. Current alfalfa cultivars typically have high levels of resistance to a suite of diseases and pests (www.alfalfa.org), including bacterial wilt (*Clavibacter michiganense* subsp. *insidiosum*), Verticillium wilt (*Verticillium albo-atrum*), Fusarium wilt (*Fusarium oxysporum* f.sp. *medicaginis*), anthracnose (*Colletotrichum trifolii*), Phytophthora root rot (*Phytophthora medicaginis*), Aphanomyces root rot (*Aphanomyces euteiches*), root-knot nematodes (*Meloidogyne* spp.), stem nematode (*Ditylenchus dipsaci*), spotted alfalfa aphid (*Therioaphis maeulata*), pea aphid (*Acyrthosipon pisum*), and others. The alfalfa research community has developed standard tests to evaluate these diseases and pests in a uniform and quantitative way (http://www.naaic.org/stdtests/index.html).

Alfalfa breeding programs are based on recurrent phenotypic selection, with or without progeny testing, to accumulate desirable alleles at high frequency into a population. Typically, an initial breeding population is screened sequentially for various diseases. Plants surviving this series of screens are planted to the field, and after several years, the most vigorous survivors are intercrossed to develop a new population. Plants may be evaluated for forage nutritive value or other traits during this cycle, and families developed from the intercrossing may be evaluated subsequently. Repeated cycles of selection, usually involving the addition of other sources of germplasm, have been conducted, with great success. Phenotypic selection is constrained by the size of breeding populations that can be evaluated in the field, by low heritability of many important traits, by genotype × environment interactions that are difficult to evaluate due to limited testing resources in most breeding programs, and phenotypic evaluations themselves that require multiple harvests per year for multiple years in order to assess persistence. All of these make selection in alfalfa less efficient than in many annual food and feed crops.

3.2. Biomass Yield

Improvement of yield in alfalfa has not been as successful as gains in other traits and has been much slower than gains seen in cereals [20–22]. Government statistics suggest yield stagnation for hay production after the 1990’s in some regions of the USA (http://www.nass.usda.gov/), results supported over the past 25 years in university-conducted variety trials in the midwestern and eastern USA and eastern Canada (D.J. Undersander, Univ. Wisconsin, pers. comm.). We compared the average biomass yield of the top five cultivars in each trial in the first post-establishment year of production. The regression of yield on the year of the trial across a total of 544 trials was close to zero (R^2 = 0.049). Most individual locations showed values close to zero (e.g., Nashua, IA, R^2 = 0.0004), although a few locations showed yield improvement over years (e.g., Marshfield, WI, R^2 = 0.23). The reasons for the location differences may be explained by an experiment that evaluated cultivars released from the mid-1950s through the 1990s [22]. This experiment showed yield improvement in environments with higher disease pressure, but not in less stressful environments, implying little genetic improvement of yield *per se* [22]. Some evidence for yield improvements has been documented, however. Cultivars from the 1980s had greater yield, but also greater inbreeding depression (based on evaluation of selfed progeny) compared to 1940s cultivars, suggesting that the yield increase was due to non-additive effects of favorable alleles accumulated from different germplasms [23].

Various explanations have been proposed for the slow improvement of yield, including the need for long selection cycles to assess multi-year persistence, the harvest of an entire aboveground plant so the harvest index can't be altered, the need to maintain forage nutritive value, selection for broad geographic adaptation and a paucity of breeders. Nevertheless, yield improvement should be possible by extending the harvesting season (early or late) as has been observed in perennial ryegrass (*Lolium perenne*) [24], or by extending the harvest interval, if nutritive value could be improved concurrently. The pooling of diverse germplasms had the associated effect of maximizing heterozygosity [23]. Maximum heterozygosity in tetraploid alfalfa has been proposed to lead to maximum yield, because of the potential for multiple allelic interactions and/or complementarity of alleles across loci [25,26]. Dominance gene action appears to play a major role for yield [27,28], suggesting that complementary alleles in repulsion phase are the cause of yield improvement. However, expecting further yield improvement from those diverse populations could be difficult to achieve because alleles for yield have been mixed among the populations, leading to difficulty in concentrating desirable alleles in one population [8,29]. A final reason for the limited gain in yield in alfalfa is that less focus is placed on yield *per se* in alfalfa than in the major grains [20,22,29]. Alfalfa yield is often selected indirectly based on evaluation of vigor on spaced plants or on short family rows rather than on measurements of yield on plots grown at stand densities used in commercial production, as is done in the major grain crops.

3.3. Hybrid Alfalfa

Heterosis for yield has been found in crosses between different alfalfa populations [30,31], suggesting that development of hybrid alfalfa could produce yield improvements. However, serious inbreeding depression and self-incompatibility have prevented the generation of inbred lines for alfalfa to date and prevented the production of single hybrids [32]. As an alternative to single cross hybrids, semi-hybrids or population hybrids could be used to capture heterosis between non-inbred parents, and this has potential to enhance alfalfa yield [21]. A modified hybrid scheme incorporating male sterility is being used by Dairyland Seeds, Inc. to produce high yield cultivars (M. Velde, pers. comm.). The source germplasm groups for developing hybrids could be the subspecies *sativa* and *falcata* [33–36]. QTL mapping in an inter-subspecies population further provided evidence of favorable alleles from *falcata* [37,38]. However, *falcata* germplasm generally contains numerous undesirable traits—e.g., low seed yield and early autumn dormancy—and the lack of improved *falcata* populations make their use in population hybrids challenging. Hybrids between dormant/semi-dormant and nondormant *sativa* germplasm could also be expected to show heterosis for yield [21]. The non-dormant Peruvian germplasm may represent a separate heterotic pool from other *sativa* germplasm [39,40], but otherwise, no clearly defined heterotic pools within *sativa* germplasm are evident [41,42]. A lack of specific combining ability effects among a large set of genotypes grown in a subtropical environment also point to the general lack of clear pre-existing heterotic groups in alfalfa [43]. The lack of existing heterotic groups in alfalfa may be due to a history of alfalfa breeding in which diverse germplasms have been repeatedly mixed together in the quest for improved disease and pest resistance. However, recent evidence from alfalfa SNP discovery research suggests little diminution of SNP variation has

occurred between wild relatives and elite breeding germplasm (Li *et al.*, in preparation). Thus, perhaps the problem is not mixing so much as insufficient differentiation of populations since domestication.

In maize, the development of the two major heterotic groups in the USA arose as a consequence of selection and enforced separation of germplasm, not due to pre-existing geographically and/or phylogenetically distinct populations [44]. Therefore, for alfalfa, we suggest that breeders create heterotic groups *de novo*. Starting with adapted, elite populations avoids the problem of germplasm like *falcata*, deficient in many necessary attributes, and enables the generation of desirable hybrids from the beginning. The method of reciprocal recurrent selection (RRS) is probably the most sensible way to develop hybrid alfalfa [45], as has recently been proposed in a timothy (*Phleum pretense*) breeding program [46,47]. An RRS program is based on two distinct populations; one population is used as the tester for the other population. Hybrid progenies are evaluated to enable selection of parental plants; the progenies are not involved in subsequent intercrossing. Intra-population crossing of superior plants results in complementary populations that perform well in hybrid combinations. Molecular markers clearly showed the effects on allele frequencies across 12 cycles of RRS in a maize breeding program [48]. Markers may be useful to form populations initially [46,47] by selecting two groups of elite breeding materials based on divergent marker genotypes to form the starting populations used to empirically evaluate hybrid potential using RRS. The value of RRS for alfalfa improvement and the utility of markers in structuring the program have not been examined, and need to be rigorously tested. But the potential of this breeding method would appear to be useful to test.

4. Genetic Dissection of Quantitative Traits Using Molecular Markers

4.1. Marker Development

Complex traits can be dissected into QTL representing individual genes (or at least, genomic regions) based on marker-trait associations [49]. Identifying QTL that underlie complex traits could augment traditional selection methods, enhancing genetic gain [9,50,51]. Currently, simple sequence repeat (SSR) markers are widely used for QTL analysis in alfalfa, most of which were derived from *Medicago truncatula* [52]. The SSR markers have facilitated genetic studies of alfalfa, including genetic map construction [53–55], comparative mapping [53,56], population structure analysis [11] and QTL identification [37,57]. However, the number of SSR markers is insufficient for the needs of fine mapping and genome-wide association studies. SNP markers are highly abundant [58] and a large number of SNP have been identified within and between two highly divergent genotypes using transcriptome sequencing [59]. A high resolution melting (HRM) technology, although not high-throughput, has been implemented to assay SNP markers in tetraploid alfalfa [60]. Recently, we sequenced 27 tetraploid and diploid alfalfa transcriptomes, including elite genotypes from commercial alfalfa breeding companies in the US. We discovered ~14,000 unique genes and ~9,000,000 SNP (Li *et al.*, in preparation). The SNPs identified in this study are publicly accessible at the Legume Information System (http://medsa.comparative-legumes.org/data/lissnp_medsa-201202.tgz).

4.2. Genetic Linkage Map Construction

A number of genetic linkage maps have been published for both diploid and tetraploid alfalfa (Table 1). Although several of the early maps randomly assigned names to linkage groups, most of the maps have tied linkage groups to chromosomes following assignments in *M. truncatula*. The length of genetic maps ranges from 234 cM to 794 cM, and many maps are marked by serious segregation distortion (SD) [61–65]. Interestingly, a large proportion of the distorted markers on F_2 linkage maps favored heterozygotes [53], suggesting over-dominant or pseudo-overdominant zygotic selection of viability genes [53]. Although mapping in diploids is simpler, mapping directly in cultivated tetraploid alfalfa should be more informative and useful for breeding applications. Alfalfa displays tetrasomic inheritance, whereby any of its four homologous chromosomes can pair with any other during meiosis. The predominance of bivalent pairing suggests that limited double reduction occurs [66]. Tetrasomic inheritance is more complex than disomic inheritance – up to four alleles can be present in a given individual and allele dosage for any allele can range from zero to four. These complexities, and especially the fact that allele dosage is typically not known for certain, challenge genetic linkage map construction for autotetraploid alfalfa. The software "TetraploidMap" [67] can incorporate both dominant (including simplex and duplex markers) and co-dominant markers into linkage maps (Table 1) [37,54,68].

Table 1. Characteristics of alfalfa genetic linkage maps.

Reference	Mapping population	Ploidy	Population size	Markers [a]	No. markers	Linkage groups	Map length	Seg. Dist.[b]	Traits of QTL identified
							(cM)	(%)	
Brummer *et al*. [61]	F_2	2*x*	86	RFLP	108	10	468	50	
Kiss *et al*. [63]	F_2	2*x*	137	RFLP, RAPD, I, M	89	8	659	--	
Kalo *et al*. [64]	F_2	2*x*	137	RFLP, RAPD, I, M	868	8	754	63	
Echt *et al*. [62]	BC	2*x*	87	RFLP, RAPD	86/61	10/7	553/603	34	
Narasimhamoorthy *et al*. [57]	BC	2*x*	130	RFLP, SSR	132	10	764	43	Aluminum tolerance [57]
Tavoletti *et al*. [65]	F_1	2*x*	55	RFLP	50/55	10/8	234/261	8.8	
Li *et al*. [53]	F_1	2*x*	190	SSR	99	8	528	24	
Li *et al*. [53]	F_1	2*x*	183	SSR	99	10	547	34	
Li *et al*. [53]	F_2	2*x*	152	SSR	90	13	391	68	Viability [53]

Table 1. *Cont.*

Reference	Mapping population	Ploidy	Population size	Markers [a]	No. markers	Linkage groups	Map length	Seg. Dist.[b]	Traits of QTL identified
Brouwer and Osborn [69]	BC	4*x*	101/101	RFLP	88 [c]	7	443	4–9	Fall dormancy and winter hardiness [69]
Sledge *et al*. [55]	BC	4*x*	93/93	SSR	286 [c]	8	624	10	
Julier *et al*. [54]	F_1	4*x*	168	AFLP, SSR	107 [c]	8	709	35-AFLP 25-SSR	
Robins *et al*. [37]	F_1	4*x*	200	RFLP, SSR	172 [c]	8	546	32	Biomass [37], morphological traits [38], winter hardiness [70], persistence [71], and self-fertility [72]
Musial *et al*. [68]	BC	4*x*	145	AFLP, SSR	203 [c]	8	794	14.3	Stagonospora root and crown rot resistance [68] and Anthracnose resistance [73]

[a] RFLP = restriction fragment length polymorphism; RAPD = random amplified polymorphic DNA; I = isozyme; M = morphological marker; AFLP = amplified fragment length polymorphism; SSR = simple sequence repeat. [b] Seg.Dist. means percentage of markers that showed segregation distortion. [c] means composite map derived from both parents.

4.3. Characteristics of Alfalfa Genetic Linkage Maps

Major QTL related to yield and morphological traits [37,38], fall dormancy and winter-hardiness [69,70], persistence [71], viability [53], self-fertility [72], resistance to *Stagonospora melioti* [68], resistance to *C. trifolii* [73], aluminum tolerance [57,74] and water use efficiency [75] have been mapped in alfalfa, primarily in tetraploid populations (Table 1). Several factors potentially limit the precision and power of mapping in tetraploid alfalfa. First, marker numbers are limited, and given that linkage maps are composed of four homologous chromosomes, saturation of the four homologues can be variable [37]. Second, relatively small mapping populations affect the precision of the linkage maps. Unlike diploid mapping populations, only 50% of individuals in a tetraploid population capture recombination events for each homolog, and only 16.7% of individuals for each homolog pair. All of the mapping populations used to date have fewer than 200 individuals (Table 1), likely resulting in an underestimation of the number of QTL and an overestimation of their effects [76,77]. Finally, if QTL are polymorphic within both parents, then the ability to detect the QTL in the population is low. Computer programs to use composite interval mapping, to analyze interactions among QTL, and to

assess genotype × environment interactions have not been developed and in some cases, the theory has not been fully developed for autotetraploids.

The major QTL identified in alfalfa to date have been located in large chromosome regions that need to be narrowed down and validated for further breeding applications. Although fine mapping QTL in an autotetraploid is not trivial, it has been done for the genetic locus *nn1* responsible for a non-nodulation phenotype [78]. This locus was subsequently cloned [79] from tetraploid alfalfa. The locus was first mapped to a candidate region using a tetraploid F_2 population of about 800 individuals, and then fine mapped on a larger F_2 mapping population of more than 2000 individuals using markers that were identified in the region using a diploid linkage map. In addition to using advanced diploid alfalfa genetic maps, further QTL localization could be accomplished through comparative mapping in other species.

Several experiments have identified genes or QTL in *M. truncatula* that could potentially be useful for alfalfa improvement. A major gene (*RCT1*) conferring resistance to anthracnose in alfalfa has been cloned in *M. truncatula* [80]. Similarly, QTL for resistance to *A. euteiches* [81,82], spring black stem and leaf spot [83] have been mapped in *M. truncatula*, but no verification of resistance in alfalfa has been documented. In addition to these disease resistances, QTL for nitrogen nutrition [84], flowering time [85] and aerial morphological characteristics [86] have been identified. A high level of synteny has been found between alfalfa and related species, including *M. truncatula* [53,56,87,88], a close relative whose genome has been sequenced [89].

4.4. Association Mapping

Quantitative trait loci can also be mapped in populations such as collections of landraces or breeding lines using “association mapping” [90,91]. This type of population would have more polymorphic QTL for a given trait and variation in more traits than a biparental mapping population, thereby providing greater inference space for the mapping exercise. Further, association mapping directly in a breeding population could provide marker information to immediately accelerate genetic gain. Like family-based QTL mapping, association mapping relies on linkage disequilibrium (LD) between a marker and the target gene (or QTL). As a consequence of historic recombination, QTL can be precisely located, compared to most family-based QTL mapping analyses, but a higher density of markers is needed to identify linked QTL because LD extends over shorter distances in the genome.

The extent of LD depends on the genetic history of a population. In a genetically diverse diploid population, LD in four genes in the lignin biosynthetic pathway rapidly decayed to less than 1kb in all cases [92]. Rapid LD decay was also observed in a CONSTANS-LIKE gene across a diverse set of tetraploid alfalfa germplasm [93]. The rapid LD decay is likely, due to the outcrossing nature of alfalfa and the large effective population size maintained in alfalfa germplasm collections. Alfalfa breeding populations that derive from a relatively narrow range of parental germplasm may have LD that is extensive enough to avoid the need for hundreds of thousands of markers in association mapping experiments. Using SSR markers, we estimated reasonably extensive LD of ~1 Mbp in a tetraploid alfalfa breeding population, although the high mutation rate of SSR markers may result in an overestimate of the LD [94]. In perennial ryegrass, *Lolium perenne*, also an outcrossing forage crop, a large proportion of the variation in flowering time in a diverse collection of natural populations and

synthetic cultivars could be explained by several AFLP markers out of a total of ~600 screened [95]. In other ryegrass populations, higher levels of LD (up to 1.6 Mbp) were observed in a cultivar derived from six related parents than in one derived from more than 300 parents [96]. To facilitate association mapping in alfalfa, the LD pattern needs to be evaluated for various populations. A computer program to assess LD between marker loci in an autotetraploid has been published [97].

An alternative to assaying thousands of markers genome-wide could be to associate SNP variation in candidate gene sequences with phenotypic variation. Sequences from a CONSTANS-LIKE gene were amplified from individuals derived from eight cultivars and two landraces [93]. Three SNP in the gene were associated with flowering date and stem height, each explaining about 4% of the genetic variation [93]. Association analysis of lignin biosynthesis genes in wild diploid alfalfa germplasm found two SNP associated with yield and stem characteristics [92]. Sequencing of the *LpHD1* gene in diverse perennial ryegrass genotypes identified one SNP associated with flowering time [98]. The limitation of the candidate gene approach, of course, is that genes other than the selected candidate genes may be the cause of phenotypic variation, and they will be missed. QTL identified from bi-parental populations could be considered as candidate regions for association mapping. This allows for validation and fine mapping QTL identified from different QTL mapping studies and various target traits. In an association mapping study, several SSR markers were found related to yield and quality in an elite alfalfa breeding population [94]. Each identified marker explained about 2–6% of phenotypic variation [94]. One of those markers, associated with yield across multiple environment, was previously identified in an unrelated bi-parental mapping population [38], suggesting that the QTL is possibly responsible for yield in a broad array of alfalfa germplasm.

Candidate genes can also be identified through gene expression profiling. Affymetrix *Medicago* GeneChip arrays have been used to evaluate heterosis for yield in alfalfa hybrids [99]. About 47% of probe sets derived from *M. truncatula* and 91% from *M. sativa* gave successful hybridization to alfalfa samples [99]. Unlike *M. truncatula*, alfalfa is highly heterozygous and allelic expression could not be measured in this experiment. Gene expression profiling can also be measured by sequencing transcriptomes [100,101] and this has led to the identification of numerous candidate genes related to stem development and cell wall composition in two alfalfa genotypes with contrasting cell wall compositions [102].

5. Using Markers in Selection

5.1. Simple Uses—Diversity Selection and Paternity Testing

Markers could be useful simply by assessing genetic diversity in a set of potential parents and selecting the most genetically divergent group as parents of the next generation (or synthetic cultivar) [46,47,103]. In these experiments, synthetics developed based on diverse parents had improved yield. However, in alfalfa, selection of parents based on marker divergence did not lead to improved yield [104]. Nevertheless, further consideration of the optimal method to apply diversity information to cultivar synthesis is warranted.

Another non-linkage based application of markers that has the potential to significantly improve genetic gain is the use of paternity testing [105]. This method could improve genetic gain by selecting

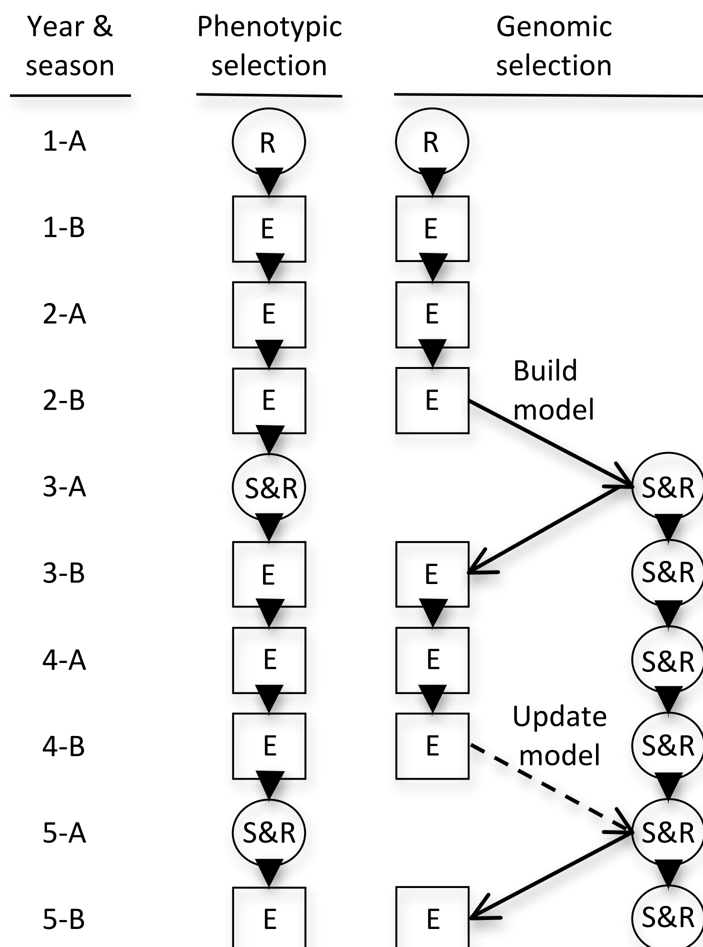
on both parents, rather than only on maternal parents, in a family selection scheme such as half-sib family recurrent selection [106]. In Riday's proposal [105], phenotypic data are obtained on each individual progeny plant, enabling estimation of both maternal and paternal breeding values. But even if data are only generated on maternal families, paternity testing could enable the selection of plants within families that have defined parentage. In red clover, paternal selection was more effective than maternal selection, although the reason for this is not clear [105]. This overlooked method could perhaps do more to improve genetic gain for a minimal cost than any other method.

5.2 Marker-Assisted Selection

One of the major problems of using unadapted germplasm is linkage drag, and markers could effectively limit the introgression of undesirable donor chromatin during the breeding process [51]. An example trait that would benefit from the use of markers is tolerance to the potato leafhopper (*Empoasca fabae*). Tolerance has been identified in several germplasm sources [107–109] and commercial cultivars with tolerance have been marketed since the late 1990s. These cultivars continue to have a modest yield drag compared to non-tolerant cultivars (M. McCaslin, Forage Genetics Int'l., pers. comm.), which could potentially be eliminated through mapping QTL for the trait and using markers to minimize the introgressed segments. Marker-assisted selection could also be used to increase the frequency of the introgressed QTL in a population by not only identifying plants carrying the QTL, but in assessing the dosage of the QTL allele, and recombining parents with higher dosages (e.g., triplex or quadriplex) to ensure a high percentage of tolerant plants in a population.

A typical phenotypic selection scheme might be sketched in thumbnail as shown in Figure 1, encompassing the generation of families, their evaluation for a suite of traits, selection of the best based on phenotypic evaluation and subsequent recombination of the selected individuals to generate a new population as well as families for the next cycle of selection. Because markers for all traits of interest are not currently available, integrating markers would likely be done in addition to field phenotypic evaluations. Thus, marker-assisted selection could be done to verify that selected individuals have particular traits—e.g., disease resistances, *etc.*—and that the dosages of the desired alleles are high. Although QTL for various traits have been mapped, considerable work needs to be done in alfalfa before marker-trait associations are sufficiently precise for MAS. Markers are most helpful if they can be identified for traits of low heritability or for traits that are difficult or time-consuming to phenotype. However, if a trait cannot be improved through phenotypic selection due to an inability to obtain accurate phenotypes, then it will not be possible to identify QTL either. MAS has not yet been applied directly to alfalfa breeding programs, with the exception of tracking transgenes.

Figure 1. A schematic comparison of phenotypic and genomic selection. For this example, we assume that two seasons are available each year, "A" typically would be winter during which intercrossing could be done in the greenhouse and "B" typically would be the growing season when phenotypes can be evaluated in the field. We begin each scheme with a recombination (R) phase to generate families for evaluation (E). Evaluations would be conducted for two years (establishment + first production year) after which selections (S) would be made and individuals recombined to generate families for the next cycle. In phenotypic selection, a single cycle would take two years. In genomic selection, phenotyping would occur in the field exactly as for phenotypic selection, but instead of making selections based on the phenotypic data, selections would be made based on genomic estimated breeding values (GEBV) obtained from a model developed from parental genotypes and family phenotypes. Following recombination, new phenotypic trials would be planted solely to provide additional data for updating the genomic selection model (Genomic selection model adapted from Jannink [110]).



5.3. Marker-Only Selection and Genomic Selection

Perhaps the greatest impact that markers could have on an alfalfa breeding program would be in marker-only selection, whereby multiple cycles per year could be realized. One of the biggest impediments to faster genetic gain in alfalfa (or any perennial crop) is the need for multi-year

evaluations that limit cycle time. This could be avoided using marker-only selection. Two strategies could be envisioned—one which selects based on markers linked to known QTL, and one that uses genome-wide markers without any knowledge of linkage relationships.

The latter approach is termed genomic selection (GS) [111–113]. In genomic selection, a genomic estimated breeding value (GEBV) is computed for each individual in the population, based on a model that includes effects of all markers for each trait. Selection is then based on the GEBV, rather than on phenotypic information. Indeed, the value of GS is that phenotypes are used only to develop and improve the model; selection is based on GEBV, which can be assessed on any plant for which DNA can be extracted and markers evaluated. As in traditional phenotypic selection, an index can be developed to simultaneously select for multiple traits. Simulation studies suggested that GS could achieve greater genetic gain per unit time for complex traits controlled by many QTL compared to phenotypic selection and MAS, depending on accuracy of the GS prediction model [110], which is affected by trait heritability, population size, LD extent, and marker number [112].

We suggest a genomic selection program in alfalfa could proceed as shown in Figure 1. An initial phase dedicated to generating phenotypic and marker data is needed, essentially paralleling a phenotypic selection program, in order to develop a GS model. Once developed, individuals can be assessed and selected, based on GEBV determined from the model. Note that phenotypic evaluation would continue as in a phenotypic selection program, but will serve not for a source of selected plants, but solely to provide additional information to improve the GS model [110]. Selection will occur on the right column based on GEBV generated by genotyping as many individuals in each generation as possible, and recombining in the greenhouse. Updating the model is necessary not only to provide additional data on marker breeding values, but also to reassess the weighting of each marker as allele frequencies change during selection. As genotyping costs decrease, GS should outperform phenotypic selection in terms of gain per unit time and cost [114].

While successful application of genomic selection has been reported in cattle [115,116], few experiments have been reported in plants [117]. For alfalfa, using GS will require models that account for its tetraploid and heterozygous nature, and this will undoubtedly make the process more complex than in a diploid species. Assessing allele dosage, rather than simply determining the presence or absence of a particular allele, will be important. Also, many markers will need to be assessed cost effectively. The most likely means to generate data at large numbers of marker loci is using genotyping-by-sequencing (GBS) [118]. Unlike diploid, inbred species, however, GBS faces several obstacles when applied to heterozygous, autotetraploid alfalfa. First, imputation of missing data at a given locus in a given individual is not trivial in an autotetraploid. In inbred diploids, imputation can be easily done based on genotypes at closely linked markers, which will be in coupling phase with the missing observation [119]. This is not the case in autotetraploids. Second, a higher depth of sequencing will be needed to generate dosage information at each locus. Nevertheless, this method is likely to be more cost effective than other assays (e.g., Illumina GoldenGate SNP arrays http://www.illumina.com /technology/goldengate_genotyping_assay.ilmn), and with the expected decrease in sequencing costs, this method should be feasible in the near future.

The alternative to GS is selection based only on a subset of markers known to be associated with QTL for the trait(s) of interest [120]. The advantage of this approach would be the limited amount of marker data needed to be generated each for cycle. The disadvantage, of course, is that a few markers

almost certainly will explain less variation for a given trait than genome-wide markers. The question is whether using a few markers, perhaps for only one or a few traits, will generate enough genetic gain to make the exercise worthwhile. Perhaps the way to apply this method would be to do a marker-only selection for one or two cycles in the greenhouse and then go back to the field and traditional phenotypic selection. At least the frequencies of desirable alleles at some loci could be increased, and this may be beneficial.

6. Conclusions

Using phenotypic selection, alfalfa breeders have greatly improved disease and pest resistances, winter tolerance and forage nutritive value, but been less successful for yield. Molecular marker can improve selection efficiency for these and other traits. Rather than simply conducting recurrent selection within broadly based germplasm, a better approach may be to develop a well documented pedigree breeding program, obtain marker data on elite clones and develop narrow-based synthetic cultivars [43]. Developing a program along these lines may enable concentration of desirable alleles for yield and other traits, and enable association mapping or genomic selection to be conducted without requiring unrealistically large numbers of markers. Marker-trait associations still need to be determined for the major disease and insect pests so that confirmation of resistances can be made among parental clones. Other uses of markers, such as paternity testing and screening for diversity during cultivar synthesis, may also improve the gain in alfalfa breeding programs.

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