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Achievements and Challenges in Improving Temperate Perennial Forage Legumes

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Achievements and Challenges in Improving Temperate Perennial Forage Legumes

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The expected move towards more sustainable crop-livestock systems implies wider cultivation of perennial forage legumes. Alfalfa (*Medicago sativa* subsp. *sativa*) is the main perennial legume in most temperate regions, especially where farm systems rely largely on forage conservation. White clover (*Trifolium repens*) and red clover (*Trifolium pratense*) are dominant in specific regions and farm systems. Although breeding progress for disease and insect resistance has been achieved, these crops have shown lower rates of genetic gain for yield than major grain crops, owing to lower breed-

ing investment, longer selection cycles, impossibility to capitalize on harvest index, outbreeding mating systems associated with severe inbreeding depression, and high interaction of genotypes with cropping conditions and crop utilizations. Increasing yield, persistence, adaptation to stressful conditions (drought; salinity; grazing) and compatibility with companion grasses are major breeding targets. We expect genetic gain for yield and other complex traits to accelerate due to progress in genetic resource utilization, genomics resource development, integration of marker-assisted selection with breeding strategies, and trait engineering. The richness in adaptive

genes of landraces and natural populations can be fully exploited through an ecological understanding of plant adaptive responses and improved breeding strategies. Useful genetic variation from secondary and tertiary gene pools of *Medicago* and *Trifolium* is being increasingly accessed. Genome sequencing projects in alfalfa and white clover will enrich physical, linkage and trait maps. Genome sequences will underpin fine mapping of useful loci and subsequent allele mining, leveraging the synteny of these crops with *M. truncatula*. Low-cost genome-wide markers generated through genotyping-by-sequencing will make genomic selection for adaptation and forage yield possible for these crops. Genetic markers will also be used for dissecting quantitative traits and developing toolboxes of functional markers for stress tolerance and other traits. Under current regulatory policies, transgenic approaches are likely to be limited to a few breakthrough traits. The key challenge for future applications of genomics technologies is their seamless integration with breeding system logistics and breeding schemes.

Keywords abiotic stress, biotic stress, diversity, forage quality, forage yield, molecular marker, plant adaptation, plant competition, seed yield, selection strategy, yield gain

I. SPECIES CULTIVATION AND UTILIZATION

A. Historical and Current Cultivation

In most temperate regions, perennial forage legumes such as alfalfa (*Medicago sativa* subsp. *sativa*), white clover (*Trifolium repens*) and red clover (*Trifolium pratense*) are historically and currently more grown than annual forage legumes such as medics, annual clovers, vetches (*Vicia* spp.) or grain legumes for biomass. They were largely introduced into European farming systems starting from the Renaissance period, where they replaced fallow and boosted thereby the forage production and nitrogen available for following cereal crops (Tarello, 1567). Annual forage legumes, however, are pivotal in a few countries, e.g. subterranean clover (*T. subterraneum*) or annual medics (*M. truncatula*, *M. polymorpha*, etc.) in Australian ley farming systems.

Compared with annual legumes, perennial legumes can offer comparable or somewhat greater agronomic and environmental advantages in terms of soil fertility, rate of nitrogen fixation per cropping year, and reduction of energy and greenhouse gas emissions (COPA-COGECA, 2007; Bues *et al.*, 2013), while providing possible additional advantages. In particular, they:

- (i) can maximize the production of feed protein per unit area, when grown in monoculture (Huyghe, 2003);
- (ii) require less pesticide and herbicide applications, and display better soil protection against erosion (Karlen *et al.*, 2007);
- (iii) can make better use of water in drought-prone areas, through more rapid regrowth at the onset of the rainy season than newly sown annuals (Porqueddu *et al.*, 2005);
- (iv) can often be cultivated also in marginal environments;
- (v) allow cost savings for tilling and sowing operations;

- (vi) are particularly suitable for energy-efficient crops such as legume-grass mixtures, in which significant below-ground nitrogen transfer takes place from legume to non-legume species (Rasmussen *et al.*, 2012);
- (vii) have remarkable flexibility of utilization (hay, silage, grazing, high-protein pellet);
- (viii) have good potential as a dual-purpose feed-energy crop (especially alfalfa; Lamb *et al.*, 2012).

In addition, deep-rooted species such as alfalfa are also suitable for reducing nitrate leaching losses and for preventing the expansion of salt-degraded soils (Russelle, 2014). Annual legumes, however, allow for much greater flexibility of crop rotations than perennials, and are preferable when outstanding levels of a climatic stress (e.g., drought) hinders the survival of the perennial crop across cropping years.

Although many perennial legumes can potentially be grown for forage, the species of actual wide interest are relatively few. Alfalfa is considered the main one, with an estimated world cropping area of about 30 million ha mainly located in North America, Europe and South America (Cash and Yuegao, 2009) and expanding use in Australia and China (Figure 1). The reported main perennial forage crop in each temperate country in terms of sown area (Figure 1) makes no distinction between pure stand and mixed stand cultivations. Two species are reported for countries whose available information (which could only partly rely on precise statistics) suggested the similar importance of two crops. The *M. sativa* complex (pooling subsp. *sativa* and the much less cultivated subsp. *falcata*) is the main crop in most countries, but white clover is preferred in some regions with a temperate climate and more intensive grazing, whereas red clover is the main species in most countries of northern Europe (Figure 1). White clover ranks as the main forage legume for mixed-species pastures in a number of countries, whereas alfalfa and red clover are mainly grown under a mowing regime. No other perennial forage species ranks as the most cultivated in any country, justifying our focus in this review on alfalfa, white clover and red clover. These species, all featuring an outbreeding mating system associated with severe inbreeding depression, also share similar breeding challenges and opportunities.

Other perennial species, however, can be quite important in specific regions. Birdsfoot trefoil (*Lotus corniculatus*), which originated in Europe and Western Asia, is widespread in several regions, particularly in North and South America. It is used for pastures or, in soils featuring low pH, also for hay or silage production (Seaney and Henson, 1970; Piano and Pecetti, 2010). Increasing interest in this species is justified by the content in its foliage of condensed tannins in concentration and structure that are suitable for reducing the degradation of forage proteins in the rumen, while displaying an anthelmintic action and minimizing the risk of bloat in grazing animals (Min *et al.*, 2003). Besides increasing ruminant production traits (Min *et al.*, 2003), condensed tannins also decrease the nitrogen excretion in the urines (Theodoridou *et al.*, 2010). A suitable content of



FIG. 1. Most-sown perennial forage crop in terms of sown area in each temperate country (with no distinction between pure stand and mixed stand surfaces). Based on country information reported in FAO's Pasture/Forage Resource Profiles (<http://www.fao.org/ag/AGP/AGPC/doc/pasture/forage.htm>) and additional indications provided by about 30 national forage crop experts that we contacted.

condensed tannins also features sulla (*Hedysarum coronarium*) (Min *et al.*, 2003), a biennial crop that is largely grown in some Mediterranean-climate countries (Piano and Pecetti, 2010). Caucasian or kura clover (*T. ambiguum*), which originated in West Asia and the Caucasus regions, occurs as diploid, tetraploid or hexaploid populations, and has become an important component of pastures in cold-prone areas of North America. Its deep tap root and the development of lateral roots confer tolerance to drought, heat, and grazing (Frame, 2005), resulting in outstanding crop persistence. Its adoption is limited by unsatisfactory establishment due to poor aerial growth of its seedling, which is a consequence of its extensive initial root growth (Taylor and Smith, 1998; Taylor, 2008). Sainfoin (*Onobrychis viciifolia*) originated in Eurasia and is cultivated for hay in areas of Western and Central Asia and the Mediterranean basin that are characterized by calcareous soils and modest rainfall (Frame 2005). Poor seed availability contributes to modest cropping of these less cultivated perennial legumes in several regions. Most of these species are unlikely to sizably increase their global importance, because forage breeding programs are expected to increasingly focus on just a few major crops whose market size could pay-off their investments in biotechnology tools (Bouton, 2007).

The genus *Medicago* originated in central and western Asia and includes over 60 species, of which two-thirds are annu-

als and one-third perennials (Quiros and Bauchan, 1988). The *M. sativa* complex includes eight diploid or autotetraploid subspecies whose evolution is still somewhat controversial (Quiros and Bauchan, 1988). Cultivated alfalfa (subsp. *sativa*), which is purple-flowered and tetraploid ($2n = 4x = 32$), underwent significant introgression from the yellow-flowered, tetraploid subsp. *falcata* in some regions of northern Europe (Muller *et al.*, 2003), producing material also described to belong to subsp. *varia*. The domestication of alfalfa is very old, when considering that Sumerian merchants traded its seed around 4000 BC (Prosperi *et al.*, 2001). Alfalfa became the main fodder crop of the Roman Empire (Columella, 1846), but disappeared from cultivation in Europe during the Barbarian invasions. It was reintroduced to Spain by the Moors in the eighth century, spread to the rest of Europe mainly from the sixteenth century onwards, was introduced in central and southern America by early settlers, and reached North America and Australasia by the beginning of the nineteenth century (Figure 2; Michaud *et al.*, 1988; Prosperi *et al.*, 2001). Nine historical germplasm introductions to North America are recognized that differ for region of origin, extent of subsp. *falcata* introgression and level of winter dormancy (Barnes *et al.*, 1977). The introduction of alfalfa to China dates back over 2000 years ago (Cash and Yuegao, 2009) and was completely independent from that in Europe and North Africa (Muller *et al.*, 2003). The *M. sativa* complex offers a range

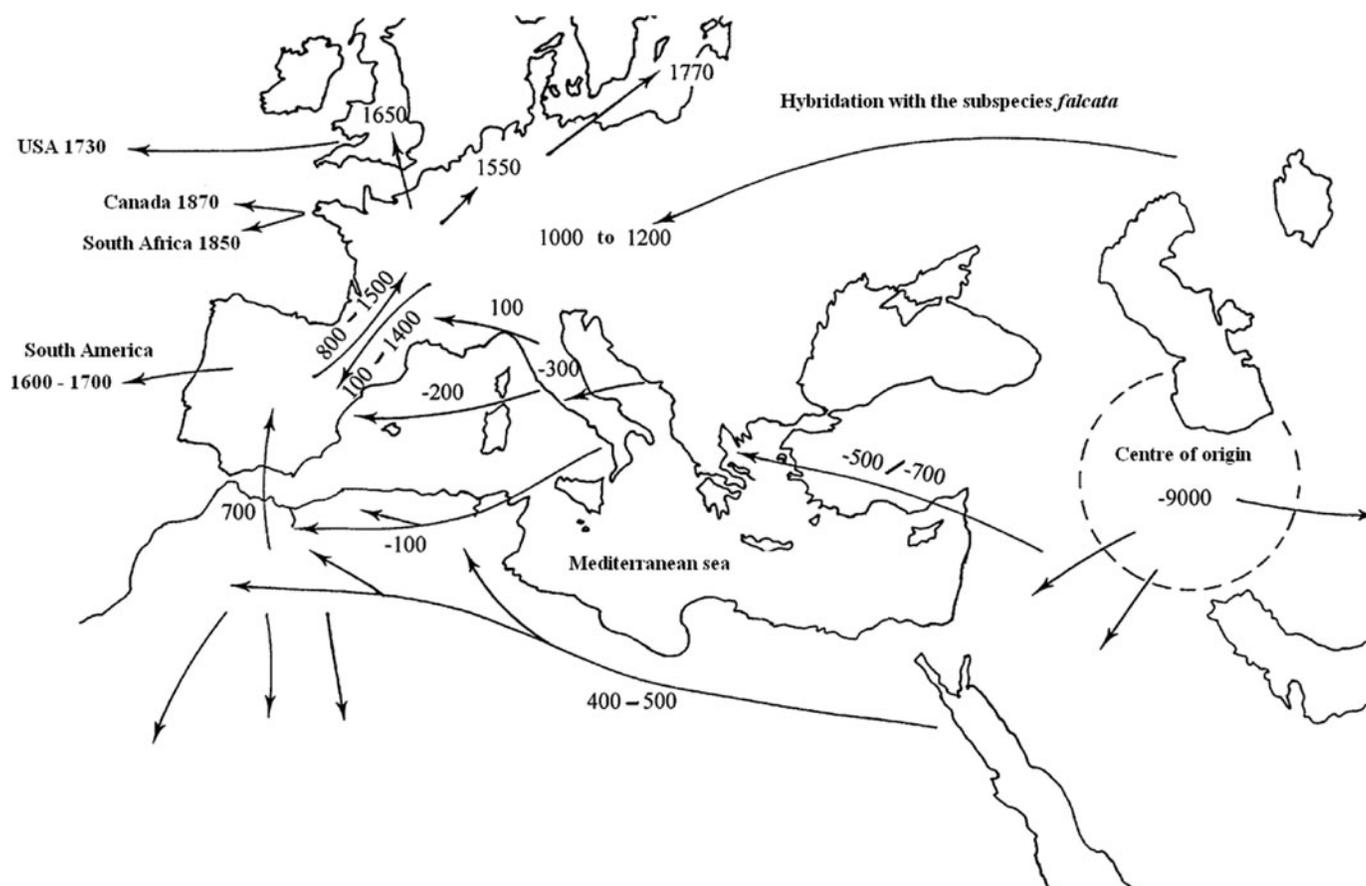


FIG. 2. The different routes and approximate dates of diffusion of cultivated alfalfa from its centre of origin; the symbol (–) correspond to BC dates (reprinted from: (Prosperi *et al.*, 2014).

of plant types from prostrate, poorly vigorous, which is typical of most subsp. *falcata* and of endemic wild populations of subsp. *sativa* found in Spain, to erect, vigorous (typical of alfalfa landraces), with implications on plant adaptation and crop utilizations (Piano *et al.*, 1996).

The genus *Trifolium* comprises over 250 species of Eurasian, American or African origin whose taxonomic, phylogenetic and evolutionary relationships are described by Zohary and Heller (1984) and updated using ITS sequences (Ellison *et al.*, 2006). White clover is an allotetraploid ($2n = 4x = 32$), stoloniferous species, which originated in the Eastern Mediterranean region, spread naturally into Europe and western Asia, and was introduced into cultivation and got naturalized in virtually all remaining temperate regions (Williams, 1987a). It was domesticated in Flanders and the Netherlands in the sixteenth century, from where it was traded in various parts of Europe (Zeven, 1991). Ecotypes of this medium-leaved type, known as *hollandicum* and an object of early breeding in the United Kingdom, and of the Ladino large-leaved type, which originated in northern Italy, were the main material introduced into cultivation worldwide (Williams, 1987a). Two additional major germplasm types used for breeding are the small-leaved type known as *sylvestre*,

which is typical of wild populations evolved under severe grazing or abiotic stress, and the large-leaved type with moderate to high cyanogenetic potential (i.e., the ability to release HCN from damaged leaves, which Ladino lacks) (Williams, 1987a; Caradus *et al.*, 1989).

Red clover is a diploid species ($2n = 2x = 14$) that is also bred as an autotetraploid following chromosome doubling through sexual ($2n$ gametes) or asexual (e.g., colchicine) methods. Its regions of origin, natural distribution, and introduction into cultivation after domestication are similar to those of white clover, but its reported cultivation from the sixteenth century onwards in areas of Belgium, the Netherlands, Italy and Spain probably followed the species domestication by the Moors (Mousset-Déclas, 1995). European landrace germplasm, which originated from early seed trade, and indigenous wild population material have been assigned to different subspecies in taxonomic classifications (Zohary and Heller, 1984), owing to the distinctly more prostrate habit, lower vigor and greater persistence of the latter material. The traditional distinction of cultivated types for northern latitudes between early-flowering, multi-harvest germplasm (also known as 'medium red' in the United States) and late-flowering, one-harvest material has been overcome by

substantial crossing between these types in breeding programs (Boller *et al.*, 2010b). Red clover is traditionally more short-lived (two- to three-year crop cycle) than alfalfa or white clover both intrinsically and because of greater susceptibility to pests and diseases, justifying the emphasis on persistence by breeding programs (Taylor, 2008). Tetraploid varieties tends towards better persistency, resistance to biotic stresses, vegetative vigor and content of proteins and sugars than diploid material (Guy *et al.*, 1989), but their diffusion is hindered by lower seed production.

B. Preferential Adaptation and Utilization

On average, alfalfa, white clover and red clover differ for adaptation to various soil, climatic, crop management and utilization conditions. The following brief indications are based on detailed information reported by Frame (2005), Sheaffer and Evers (2007) and in the books edited by Baker and Williams (1987) and by Hanson *et al.* (1988). Each species, however, is characterized by wide genetic variation for adaptation to specific growing conditions (Williams, 1987a; Taylor, 2008; Annicchiarico *et al.*, 2010) that can be exploited to widen substantially the species adaptation and utilization.

White clover and red clover are moderately tolerant to acid soils (minimal pH > 5.5; optimum pH 6.0 - 6.5), while being unadapted to even slightly alkaline soils. Alfalfa has optimal pH in the range 6.6 - 7.5, requires soil pH well-above 6.0, and is moderately tolerant to alkaline soils. All three species are sensitive to aluminum toxicity arising in acid soils, which limits root growth and nutrient uptake and requires lime application or the development and adoption of tolerant germplasm (e.g., Khu *et al.*, 2012).

Drought tolerance is high in alfalfa because of its deep rooting system, intermediate in red clover, and modest in white clover, whose taproot hardly survives the first production year and is progressively replaced by shallow roots originating from stolon nodes. All three species have modest tolerance to saline soils, and similar optimum growing temperatures (between 20 and 25°C). Within-species variation is wide for frost tolerance in all species, while being wide in alfalfa, moderate in red clover and modest in white clover for drought and salt tolerance (Abberton and Marshall, 2005; Annicchiarico *et al.*, 2011). In general, frost tolerance is associated with the level of cold stress under which the parent germplasm evolved or was selected, and is enhanced by a hardening period with low positive temperatures preceding the frosts. Alfalfa varieties are classified as a function of their autumn dormancy, i.e., the ability to arrest vegetative growth in autumn, a trait associated with tolerance to winter frost (although not being the only determinant of it: Brummer *et al.*, 2000). Specifically-adapted alfalfa material may survive low temperatures of -25°C in North America or Siberia, and high temperatures around 50°C in North Africa or California.

White clover is ordinarily cultivated in mixed stands with one or more forage grass species, whereas alfalfa and red clover may be cultivated in either pure stands or mixed stands. Pure stand cropping ensures forage with high protein content, no need of

nitrogen fertilization, and high levels of fixed nitrogen available for the following crop. However, mixed cropping can provide the following advantages relative to a pure stand: (i) higher yield due to complementarity between species for growing season and use of resources (e.g., Nyfeler *et al.*, 2009), including below ground transfer of biologically-fixed nitrogen from legumes to grasses (Rasmussen *et al.*, 2007); (ii) lower weed invasion (unless applying chemical weed control); (iii) more balanced energy/protein balance; and (iv) reduced risk of bloat for grazing animals (high risk being associated with grazing large amounts of leafy legumes). Obtaining a balanced proportion of the legume and grass components can be difficult, though. Legume-grass competition dynamics are markedly affected by species and variety of legume and grass companions, timing and extent of nitrogen fertilization, and frequency of defoliation (Harris, 1987; Chamblee and Collins, 1988). Erect species such as alfalfa and red clover can withstand grass competition for light and nutrients better than white clover, but within-species variation for competitive ability and compatibility can be high (especially in white clover: Annicchiarico, 2003).

Forage legumes are of considerable importance for ruminant feeding, owing to their high protein content, high buffering effect, which reduces the risk of acidosis, and fairly high energy content (Frame, 2005; Barnes *et al.*, 2007). Combined with perennial grasses or corn silage, they provide a diet which is suitable for highly-productive animals, with little requirements of energy or protein supplementation. Forages can be fed to animals through grazing or after being mown (as hay, silage, or fresh forage). Grazing is widespread in extensive crop-livestock systems, where it minimizes feeding costs. Tolerance to grazing is important for stand persistence in these systems, especially when grazing is continuous, with high stocking rates and/or performed by sheep (Frame, 2005). Because of its stoloniferous habit, white clover is less affected by close, intense grazing than alfalfa and red clover, whose regrowth requires regeneration of new buds from the crown. However, all three species display remarkable within-species variation for adaptation to severe grazing that is associated with specific morphological traits and tends to be inversely related to high yielding ability under a mowing regime (Evans *et al.*, 1992; Taylor, 2008; Annicchiarico *et al.*, 2010). The risk of bloating can be minimized by mixed cropping with grasses. Although white clover flowers contain condensed tannins, they are at insufficient levels to influence proteolysis in the rumen, and future efforts need to focus on foliar expression of condensed tannins (Burggraaf *et al.*, 2008).

Forage conservation through hay is energy-efficient, but high leaf losses may occur under unfavorable climatic conditions which reduce forage quality. Silage is preferable in these conditions, but may imply high proteolytic losses because of the lack in these legumes of condensed tannins that would bind the proteins and prevent their degradation. Ensiling wilted forage containing more than 40% dry matter improves silage preservation and limits proteolysis (Demarquilly *et al.*, 1998). Red clover undergoes much lower proteolysis during ensiling than

alfalfa or white clover, because of its polyphenol oxidase activity (Sullivan and Hatfield, 2006). Red clover can also provide increased amounts of healthy compounds in the milk, such as polyunsaturated fatty acids (Dewhurst *et al.*, 2003).

Dehydration, by which the forage is shortly dried at between 250°C and 700°C, is an alternative conservation strategy for producing high-quality forage that is mainly used for alfalfa in southern Europe. From the environmental standpoint, its relatively high energy cost (Gallego *et al.*, 2011) can be compensated by various advantages associated with the introduction of a perennial legume in a cereal-based cropping system, such as increased soil structure and fertility, lower nitrogen leaching, and easier weed management (e.g., Meiss *et al.*, 2010).

Alfalfa may have an interest also for energy production, if devised as a dual-purpose crop. In particular, leaves and stems could be separated at harvesting, using the former as a high-protein feedstuff, and processing the stems to produce electricity (by combustion) or biofuel [by ethanol (fermentation) or bio-oil (pyrolysis)] (DeLong *et al.*, 1995). The optimum crop management and plant type for this use are different from those for conventional forage production (Lamb *et al.*, 2007).

II. ACHIEVED BREEDING GAINS

Forage yield undoubtedly is the main objective of breeding programs for perennial forage legumes (Abberton and Marshall, 2005; Vogel and Lamb, 2007; Boller *et al.*, 2010a). Yield selection can be for maximum short-term yield, or through increased persistence (intrinsic or due to stress tolerance) to provide high long-term yield. Achieved breeding gains are bet-

ter estimated by specific experiments rather than by statistical data for on-farm yields in a given region, since the latter are not precisely monitored, confound genetic and management improvement, and may be subjected to wide climatic variation. Experimentally-based estimates of historical rates of genetic gain arising from breeding are well-documented only for alfalfa yields in the United States, for which they consistently suggest a yearly progress around 0.25% (Table 1). The multi-location assessment by Lamb *et al.* (2006) provides further insight, showing rates near to 0.50% in two sites with high pressure of pests, and nearly no gains in two other sites featuring modest biotic stresses. This highlights the success derived by the high focus of U.S. breeders on pest tolerance (Bouton, 2012), as well as the substantial failure of breeding to improve the crop yield potential. The scant information available suggests lower rates of genetic gain for alfalfa yield in Europe than in United States, as well as lower rates of annual yield improvement for alfalfa than for white clover in New Zealand (0.60%) and red clover in the United States (0.53%) (Table 1). Even in the most favorable cases, however, yield gains for perennial forage legumes are well below the value of 1.4% estimated for maize forage yield in the United States (Lauer *et al.*, 2001). They also tend to be lower than those reported for major grain crops, e.g., wheat, whose rates in the United States ranged from 1.4% to 0.4% in various experiments reported by Cox *et al.* (1988) and by Green *et al.* (2012). The higher rates of yield improvement for these annual crops can be fully accounted for by the combination of the following: (i) higher breeding investment; (ii) shorter selection cycles; (iii) the possibility to exploit more efficient

TABLE 1

Estimated rate of genetic gain from plant breeding, for forage yield under a mowing regime and forage quality traits of alfalfa, white clover and red clover

Species	Trait	Region	Lapse of cultivar release	No. of tested cultivars	Genetic gain (%/year)	Source
Alfalfa	Forage yield	USA	1940–1995	14	0.26	Lamb <i>et al.</i> , 2006 ^a
Alfalfa	Forage yield	USA	1945–1985	14	0.22	Holland and Bingham, 1994
Alfalfa	Forage yield	USA	1971–1981	Not given	0.26	Hill Jr <i>et al.</i> , 1988
Alfalfa	Forage yield	France	1980–2000	30	~0.00	Tabel and Allertit, 2005
Alfalfa	Forage yield	France	1996–2012	22	0.11	GEVES (http://www.herbe-book.org/) ^b
Alfalfa	Forage yield	Italy	1956–2006	11	0.15	See Table 2
White clover	Forage yield	New Zealand	1920–1990	110	0.60	Woodfield and Caradus, 1994
Red clover	Forage yield	USA	1953–1987	4	0.53	Smith, 2001; Riday and Krohn, 2010b
Alfalfa	Digestibility; protein%; NDF	USA	1940–1995	16	~0.00	Lamb <i>et al.</i> , 2006
Alfalfa	Protein%	France	1980–2000	30	0.02	Tabel and Allertit, 2005
Alfalfa	Protein%	France	1996–2012	22	~0.00	GEVES (http://www.herbe-book.org/) ^b

^aBased on Fig. 1 data of this source, averaged across four test sites. Genetic gains vary from 0.52 in a site with high biotic stress, to nil in a site with limited biotic stress.

^bComputed from VCU (Value for Cultivation and Use) data in different variety registration trials adopting a common control variety.

TABLE 2

Three-year forage yield in the region of adaptation and in a contrasting region, for Italian commercial ecotypes and varieties from Lombardy and from Romagna or Umbria, and estimated rate of genetic gain from breeding in each region. Based on data by Annicchiarico *et al.* (2012)

Area of origin or selection	Cultivar	Year of release	Forage yield (t/ha)		Gain (%/year) ^a
			Lodi (Lombardy)	Perugia (Umbria)	
Lombardy	Ecotype Cremonese ^b	—	19.61	28.02	—
Lombardy	Lodi	1987	19.77	28.74	0.03
Lombardy	Costanza	2006	21.27	27.33	0.17
Romagna/Umbria	Ecotype Romagnolo and Perugino ^c	—	17.34	29.30	—
Romagna/Umbria	Prosementi	1973	16.65	29.16	-0.03
Romagna/Umbria	Classe	1997	16.50	29.49	0.02
Romagna/Umbria	Casalina and Cuore Verde	2004	11.88	31.14	0.13

^aRelative to the relevant ecotype in the region of adaptation, assuming 1956 as the reference year for the start of any breeding program in both regions. An overall average gain of 0.15% is estimated by averaging the gain in each region of the most recent variety relative to the relevant ecotype.

^bAverage of two farm landraces.

^cAverage of three farm landraces.

breeding schemes allowing for the selection of hybrids or pure lines (unlike perennial forages, whose outbreeding mating system, severe inbreeding depression and available male sterility systems allows for synthetic variety breeding or, at most, for partly hybrid varieties); (iv) the possibility for grain crops to capitalize on the harvest index (whereas forage yield is just the result of photosynthetic activity, itself a function of intercepted photosynthetically active radiation); and (v) the possibility for annual crops to tune flowering date and crop duration so as to escape abiotic stresses (e.g., drought).

The lower rate of forage yield improvement for alfalfa relative to white clover and red clover suggested by results in Table 1 is a little surprising, given the similarity of these species in terms of breeding challenges and selection schemes. Actually, the estimate for red clover in the United States may suffer from limited inference space (being based on a single experiment including just a few cultivars), whereas a critical review of yield gains for white clover in New Zealand suggests some uncertainty and possible overestimation of rates that had previously been reported (Parsons *et al.*, 2011). However, contributing reasons for lower breeding progress in alfalfa than in white clover and red clover might be the tetrasomic inheritance and the higher extent of non-additive genetic variance that arises from gene interaction in alfalfa (Bingham *et al.*, 1994). In addition, slower breeding progress can be expected when breeding a species with a long history of cultivation and adaptation to a target region, such as alfalfa in Europe. Indeed, landraces or old cultivars derived from landraces showed forage yields comparable with those of modern varieties, when they were grown in the European region in which they evolved (see Annicchiarico [2006a] for alfalfa, and Boller *et al.* [2010b] for red clover). Such good performance has justified the presence in the Italian seed market

of alfalfa farm landraces (grouped into several geographically-based commercial ecotypes) until 2003. A comparison of alfalfa landraces and varieties released in different years, for two sets of material originated from, or selected in, two contrasting regions of Italy is shown in Table 2. Gains from breeding are manifest only for comparisons that are performed in the target region of the material (e.g., 'Costanza' > 'Lodi' > 'Ecotype Cremonese' in Lombardy, but not in Romagna/Umbria), highlighting the importance of the evaluation conditions when assessing such gains. In addition, the rate of annual breeding progress, averaging 0.15% when comparing the most recent variety vs. the relevant ecotype in each region, tends to be greater in recent years than in early years (e.g., it would rise to 0.40% for Lombardy between 1987 and 2006, based on the comparison of 'Costanza' vs. 'Lodi').

The rates of yield gain reported in Table 1 refer to assessments under a mowing regime. However, persistence data reported by Smith Jr *et al.* (2000) and by Pecetti *et al.* (2008) suggest that the rate of alfalfa yield progress might be much greater when considering selections developed for grazing animals. Breeding progress has been noticeable also for alfalfa tolerance to specific pests such as bacterial wilt (Elgin *et al.*, 1988), *Verticillium* wilt (Toth and Bakheit, 1983), anthracnose (Elgin and Ostazeski, 1985), stem nematode (Elgin *et al.*, 1988) and potato leafhopper (Ranger and Hower, 2001), intrinsic persistence of red clover (Smith, 2001; Taylor, 2008), and tolerance of white clover to low temperatures, grazing (Rhodes *et al.*, 1994), root-knot, and clover cyst nematode (Mercer *et al.*, 2000).

Forage quality has increasing interest for breeders of perennial forage legumes, particularly with respect to high digestibility for alfalfa and red clover, and proportion of non-degradable protein (i.e., the protein that escapes rapid microbial

degradation in the rumen followed by excretion of nitrogen in the urine) and lower bloating potential in all species (Smith *et al.*, 1997). Other traits might have special importance for specific crop utilizations (e.g., low rate of proteolysis for silage, or high protein content for production of high-protein feedstuff). The information summarized in Table 1 suggests that no overall variation for forage digestibility and contents of protein and neutral detergent fiber (NDF, associated with rate of forage ingestion by animals) has arisen from alfalfa breeding. However, successful selection for all of these traits has been performed and made available by specific “high-quality” alfalfa cultivars (Hall *et al.*, 2000).

Forage quality is being improved also through transgenic varieties. Transgenic alfalfa cultivars featuring higher digestibility as a consequence of modified lignin composition (Guo *et al.*, 2001b) may be available in the USA within a few years (with high potential interest also for the dual-purpose, forage-energy crop: Dien *et al.*, 2011). Transgenic alfalfa, white clover and red clover varieties with activated accumulation of condensed tannins in the leaves could be produced (Hancock *et al.*, 2012) with the aims of drastically increasing the proportion of non-degradable proteins and reducing the risk of bloat.

There is little information available on changes in seed yield of perennial forage legumes as a consequence of plant breeding. Over 20% improvement was reported for seed yield of white clover in a single cycle of selection (Widdup *et al.*, 2004). Markers have also been shown to be effective in selecting higher seed yielding individuals in complex breeding populations (Barrett *et al.*, 2009). However, the trend towards improvement of commercial varieties as a whole is not known. Italian landraces and modern varieties of alfalfa exhibited no difference for seed yield (Annicchiarico *et al.*, 2007). Tetraploid red clovers have at least 20% lower seed yields relative to diploid material (Boller *et al.*, 2010b), severely limiting their commercial viability. Grazing-type alfalfa varieties tend to have lower seed yield than conventional varieties (Smith Jr and Bouton, 1989), especially when largely bred from subsp. *falcata* germplasm (Pecetti *et al.*, 2008). The importance of seed yield for commercial success of forage legume varieties should not be overlooked. For example, the French white clover cultivar ‘Crau’ displayed outstanding forage yield across a number of testing conditions (Caradus and Woodfield, 1997; Annicchiarico, 2012) but has been withdrawn from the seed market, presumably because of its very low seed yielding ability (Annicchiarico, 2012). ‘Tillman’ is another example of a white clover cultivar which failed commercial success because of low seed yield.

III. GENETIC RESOURCES

A. Alfalfa

Cultivated alfalfa belongs to subspecies *sativa* of the *M. sativa* complex, which belongs to the section *Falcago* and com-

prises the following eight subspecies (Quiros and Bauchan, 1988):

- (i) *sativa*, autotetraploid with purple flowers and coiled pods;
- (ii) *falcata*, diploid or autotetraploid with yellow flowers and sickle-shaped pods;
- (iii) *coerulea*, diploid with purple flowers and coiled pods;
- (iv) *glutinosa*, autotetraploid with yellow flowers and coiled pods;
- (v) *glomerata*, diploid or autotetraploid with yellow flowers and coiled pods;
- (vi) *varia* and *hemicycla*, with variegated flowers, derived from hybridization with *falcata* of *sativa* and *coerulea*, respectively;
- (vii) *polychroa* and *tunetana*, derived from hybridization of *sativa* with *glutinosa* and of *sativa* or *coerulea* with *glomerata*, respectively.

These subspecies freely intercross and produce fertile progenies at a given ploidy level. The occurrence of unreduced gametes in the diploid populations enables genetic exchanges between ploidy levels. Extensive descriptions of the *Medicago* genus were carried out by Sinskaya (1961) and by Lesins and Lesins (1979). The most recent and comprehensive consideration of the genus by Small (2011) resolves many previous inconsistencies and provides the best classification of all species included in the *M. sativa* complex. Polyploidy generates increased size of vegetative and reproductive organs, justifying the selection of polyploidy forms for cultivation. Molecular data suggest that cultivated alfalfa lost about 30% of its genetic diversity compared to wild populations during domestication (Muller *et al.*, 2006), although transcript profiling of a limited number of wild diploid and cultivated tetraploid genotypes suggested allelic loss closer to 9% (Li *et al.*, 2012).

The genetic resources of prominent agricultural interest includes wild and cultivated populations of subsp. *sativa* and *falcata* (Prosperi *et al.*, 2001). These subspecies also differ for root system, which is tap-rooted in *sativa* and fasciculate in *falcata*. Some *falcata* populations exhibit rhizomatous or creeping root systems. *Falcata* populations that are present in a wide geographic region stretching from eastern France to Siberia and from the Black Sea coast of Bulgaria to St. Petersburg are very tolerant to cold winters. Conversely, subsp. *sativa* populations, which occur in areas of the Mediterranean basin, the Near and Middle East, the Caucasus and central Asia, are more adapted to temperate climates. Some populations from both subspecies may survive severe drought periods. Wild populations mostly display prostrate growth habit as an adaptive trait for limiting grazing losses, whereas cultivated populations usually are erect as a result of natural selection for ability to compete for light and anthropic selection for plant vigor and ease of forage harvesting. The sickle-shaped pods of *falcata* favor pod shattering and seed dispersal, unlike the coiled pods of *sativa*. Thus, erect growth habit, coiled pods and polyploidy probably are the three major traits involved in the domestication of alfalfa.

The separation of the different subspecies and of wild and cultivated germplasm was probably favored by geographic isolation. In addition, restricted pollen flow and natural selective pressures on the wild compartment can limit gene exchanges from cultivated to wild populations (Jenczewski *et al.*, 1999). Gene flow does occur, however, as shown between wild populations (called Mielgas) and cultivated populations of *M. sativa* in Spain on the basis of molecular (Jenczewski *et al.*, 1999) and morphophysiological observations (Prosperi *et al.*, 2006). A large region of sympatry from central Europe through central Asia has enabled hybridization between *sativa* and *falcata* to produce the widely distributed hybrid subspecies *varia*. Much cultivated *sativa* germplasm includes varying degrees of *varia* germplasm. The introgression from *falcata* has improved the cold tolerance of *sativa* populations, as found in French Flemish-type landraces. Because of the prevalence of subspecies gene flow and the similarity between some wild populations of *sativa* and *falcata*, genetic resources for breeding purposes are more effectively classified based on morphophysiological traits than subspecies status (Piano *et al.*, 1996).

Although autopolyploidy may evolve towards disomic inheritance (Soltis and Soltis, 1993), autotetraploidy in alfalfa has been stable throughout its long cultivation history. Indeed, autotetraploidy is a positive trait to widen the species environmental adaptation, because it offers to plants the capacity to host a large number of mutations (Parisod *et al.*, 2010) and conserve several alleles that generate homeostasis under stressed conditions (Gallais, 2003). In addition, autotetraploidy favors the maintenance of heterosis and of large genetic variance between individuals and allelic diversity within individuals (Gallais, 2003). The analysis of segregating molecular markers (Julier *et al.*, 2003) has confirmed alfalfa tetrasomic inheritance with no trend towards preferential pairing of chromosomes.

Genetic diversity used for breeding is mostly based on cultivated populations. Studies of genetic structure for major agronomic traits have showed that within-population diversity is much larger than between-population diversity. The estimated variance of the former relative to the latter for forage yield and its components was about 10- to 30-fold larger for landraces and about 7- to 15-fold larger for varieties of Italian origin (Annicchiarico, 2006a), and even greater for material mainly of French origin (Julier *et al.*, 2000). In the same materials, intra-population variation proved wider than (Annicchiarico, 2007a) or mostly comparable (Julier *et al.*, 2000) to between-population variation for leaf/stem ratio or other traits associated with forage quality, while being larger for seed yield and its components in a world collection of populations (Bolaños-Aguilar *et al.*, 2000). These findings have important implications for strategies of genetic base establishment and preliminary screening by breeding programs, which need be consistent with the primary target of exploiting intra-population diversity. Such wide within-population variation may have several contributing reasons, such as: (i) the reproductive system encouraging cross-pollination, and the fact that pollinating insects are able to travel

several hundred meters (St. Amand *et al.*, 2000); (ii) population exchanges among farmers, regions or countries, which have always been important (Julier, 1996); (iii) autotetraploidy, which increases the number of alleles per locus and the number of allelic combinations. High within-population diversity can be beneficial for coping with varying levels of stresses that affect perennial species.

Landrace germplasm of alfalfa (or other perennial species more generally) is specifically adapted to environmental conditions under which it evolved. Specific adaptation may be developed not only for stress environments but also for favorable ones, as shown by some Italian alfalfa landraces (Annicchiarico and Piano, 2005), and is frequent also in varieties obtained from landrace germplasm and/or selected in specific conditions (Annicchiarico *et al.*, 2012). All of that has a bearing on strategies for locating, evaluating and exploiting genetic resources, highlighting (i) the particular importance of collecting information for locating potentially useful material, (ii) the strict dependence of the estimated germplasm value on the specific evaluation conditions, and (iii) the appeal of specific adaptation strategies to fully exploit the breeding potential of some populations (Annicchiarico, 2007c).

The use of wild populations in breeding programs has been fairly limited. The main reason is the genetic load carried by wild populations (low forage yield potential, prostrate growth habit and possibly diploidy). In addition, the huge diversity available in the cultivated pool has usually been sufficient for locating useful genetic resources for major breeding targets or new traits. Wild populations have proved very useful for improving the tolerance to severe grazing (Pecetti *et al.*, 2006) and to potato leafhopper (obtained by introgressing the glandular hair trait: [Kitch *et al.*, 1985]). In addition, the hybridization of *sativa* and *falcata* populations can display large heterosis (Riday *et al.*, 2003).

Feral alfalfa populations are frequent along infrequently mowed, non-fertilized roadsides of cultivated areas. They have not been extensively studied (Bagavathiannan and Van Acker, 2009) but may offer better tolerance to specific stresses while being phenotypically similar to the cultivated pool. For example, feral alfalfa germplasm from Manitoba (Canada) had high winter hardiness, rhizome production, and less erect growth habit than cultivated alfalfa (Bagavathiannan *et al.*, 2010). Crosses between *M. sativa* populations and other *Medicago* species, annual or perennial, have been reported (Quiros and Bauchan, 1988), but little breeding effort has been devoted to this direction.

Genetic resources are conserved in numerous gene banks (National Plant Germplasm System in the United States, Australian *Medicago* Genetic Resource Centre in Australia, ICARDA in Syria, Vavilov Institute in Russia, etc.). In Europe, the European Cooperative Programme for Plant Genetic Resources (ECPGR: <http://www.ecpgr.cgiar.org>) coordinates the maintenance of resources own by national banks, while the web-based EURISCO catalogue holds alfalfa information from gene banks of 25 European countries (<http://eurisco.ecpgr.org>). Wild

perennial species are poorly represented or not represented in germplasm collections, especially those from the center of origin of the genus and from Asia. Diploid forms of the *M. sativa* complex belonging to subspecies *glomerata* and *coerulea* are also under-represented in gene banks, where the large majority of the accessions are tetraploid *M. sativa* populations.

B. White Clover

The center of origin of white and red clovers is believed to be in the Eastern regions of the Mediterranean, where the greatest variety of species is found (Vavilov, 1926). White clover is a tetraploid species with disomic inheritance and is likely to be of hybrid origin (Ellison *et al.*, 2006). Originally in section Lotoidea (Zohary and Heller, 1984), on the basis of DNA sequencing phylogeny it has now been placed in the section *Trifolium* along with its closest relatives (Ellison *et al.*, 2006). These species are very diverse, ranging from annuals to long-lived perennials (Table 3), and offer potential genetic resources for genome elucidation and improvement (Williams and Nichols, 2011). White clover is native to and widespread in Europe, Eurasia, the Middle East and North Africa, and was introduced as a cultivated species in many countries of the world. *T. occidentale*, a diploid ($2n = 2x = 16$) perennial species, is considered to be a likely ancestral diploid parent of white clover (Ellison *et al.*, 2006), while the other diploid parent has been proposed as *T. nigrescens* or *T. pallelescens* but has not yet been confirmed (Ravagnani, unpublished data). *T. occidentale* is also being developed as a model species for genetic and genomic research relating to the white clover complex (Williams *et al.*, 2009).

The genetic resources in *Trifolium*, and their utilization in plant breeding of red and white clover and other clover species, has been the subject of several recent reviews (Abberton and

Marshall, 2005; Abberton and Thomas, 2011; Ravagnani *et al.*, 2012). White clover exhibits considerable adaptive variation between populations (Williams, 1987a), along with high levels of phenotypic plasticity and intra-population variation. Within-population variance was 2- to 5-fold larger than between-population variance for forage yield and its components, and mostly even larger for seed yield components, in a set of Ladino landraces and wild populations collected and evaluated in their region of origin (Annicchiarico and Piano, 1995). Lack of genetic variation *per se* is not a major issue in the breeding of white clover (Abberton and Thomas, 2011). The use of *ex situ* genetic resources has been critical in the development of new varieties. Extensive ecotype collections are available in many countries, providing an array of adapted plant types of different leaf size for direct exploitation or as material for crossing with Ladino types. Traditionally, novel plant material has been obtained by plant breeders through plant collection expeditions to geographic areas where desired traits may be found (Abberton and Marshall, 2010). Plant breeders also use genebank collections as a source of genetic material. The European Central white clover database (www.ecpgr.cgiar.org) contains passport data on 1350 accessions of white clover stored in 14 European genebanks as well as a portal for access to other genebanks and collections outside Europe, as described in detail by Abberton and Thomas (2011). The Margot Forde Germplasm Centre (New Zealand's national genebank for grassland plants) holds over 25,000 white clover accessions.

Ecotypes have been the backbone of white clover breeding, owing to the outstanding agronomic value that some of them may display. In Italy, for example, some Ladino landraces exhibited higher forage yield than Italian or foreign large-leaved varieties (Annicchiarico, 1993), whereas non-Ladino, medium-leaved wild populations performed comparably with medium-leaved varieties both under mowing and under grazing managements (Annicchiarico, 2012). Wild populations of Swiss origin displaying outstanding winter survival and early spring growth (Collins *et al.*, 1991) provided the genetic base for recent varieties in UK. In the southern United States, naturalized ecotypes have been used as the base germplasm to increase persistence and performance under grazing, producing successful cultivars such as 'Durana,' based strictly on ecotype material, and 'Patriot', a population cross of an ecotype with a Ladino type. White clover wild populations collected from the USA displayed better yield and persistence than New Zealand and U.S. Ladino types when grown in Eastern USA (Widdup *et al.*, 1995).

For a small number of important traits, significant programs of interspecific hybridization have been undertaken using crop wild relatives. These have also been aimed at identifying the progenitor species of white clover (Chen and Gibson, 1971; Chen and Gibson, 1972). A comprehensive summary of the potential value of crop wild relatives by Williams and Nichols (2011) includes closely related species that are both annual and perennial and of different ploidy (2x, 4x and 6x). These include

TABLE 3

The species closely related to white clover (section *Trifolium*), after Williams and Nichols (2011)

Species	Ploidy	Annual/perennial
<i>T. nigrescens</i>	2x	Annual
<i>T. occidentale</i>	2x	Perennial
<i>T. uniflorum</i>	4x	Perennial
<i>T. ismocarpum</i>	2x	Annual
<i>T. thalii</i>	2x	Perennial
<i>T. pallelescens</i>	2x	Perennial
<i>T. retusum</i>	2x	Annual
<i>T. suffocatum</i>	2x	Annual
<i>T. parnassi</i>	2x	Perennial
<i>T. cernuum</i>	2x	Annual
<i>T. glomeratum</i>	2x	Annual
<i>T. ambiguum</i>	2x, 4x, 6x	Perennial
<i>T. montanum</i>	2x, 4x	Perennial

T. nigrescens, *T. ambiguum* and *T. uniflorum* as the main species exploited for genetic improvement, whilst many other species such as *T. pallescens* have been used for cytology studies and to explore relationships within the *Trifolium* genus. *T. occidentale* has been developed as a model species for genetic and genomic research relating to the white clover complex (Williams *et al.*, 2009). The potential role of interspecific hybridization using these wild relatives in the genetic improvement of white clover has been discussed by Abberton and Marshall (2005) and Ravagnani *et al.* (2012).

With respect to white clover, several major programs have been undertaken, predominantly in New Zealand and the UK, aimed at introducing important traits into white clover (Williams, 2014). Crosses have been made between white clover and the diploid annual species *T. nigrescens* (ball clover) ($2n = 2x = 16$), a profusely flowering species that has high seed yield relative to white clover and good nematode resistance, to introgress cyst nematode resistance (Hussain *et al.*, 1997), root-knot nematode resistance (Pederson and Windham, 1989), and high inflorescence production and seed set (Marshall *et al.*, 1995) into white clover. For this latter breeding target, a backcrossing program has produced selection lines that combine the agronomic characteristics of white clover with increased seed yield due to increased inflorescence production (Marshall *et al.*, 2008). Tetraploid *T. ambiguum* material has been used for introgressing the rhizomatous trait into white clover, producing selection lines with greatly enhanced drought tolerance in comparison with its white clover parent and control varieties (Marshall *et al.*, 2001; Widdup *et al.*, 2003), as well as improved forage quality characteristics (Marshall *et al.*, 2004). The potential of interspecific hybrids between white clover and *T. uniflorum* to improve drought tolerance has also been carried out (Williams, 2014). Partially fertile backcrosses to *T. ambiguum* were also reported with different ploidy levels (Williams *et al.*, 2006). More recently, interspecific hybridization between white clover's putative diploid progenitor species has been used to create distinct new populations of value to plant breeding (Williams *et al.*, 2012).

C. Red Clover

Red clover is a natural diploid but has a large number of synthetic autotetraploid cultivars. The phylogenetic relationship between red clover and related species is not as clear as for white clover (Ravagnani *et al.*, 2012). Most studies are based on cross-compatibility and chromosome pairing in hybrids, but a complication is represented by the fact that red clover is the only species with $n = 7$ amongst its closest relatives in the section *Trifolium* (Ellison *et al.*, 2006). The classification of red clover has been reviewed by Boller *et al.* (2010b).

As described in Section I, cultivated red clover can be classified according to ploidy level and earliness of flowering. Tetraploid varieties show lower fertility (seed formation) than diploids, probably due to both multivalent formation and reduced pollination. Landraces have historically been developed

and are now conserved (recently mainly *ex situ*) in many countries, preserving the diversity that often served as a starting point for subsequent breeding programs (Boller *et al.*, 2010b). Landraces play a more prominent role for red clover than for white clover breeding, owing to the long cultivation history of the species and to the relatively strong differentiation between cultivated and wild (spontaneous) forms. Compared to landraces, wild populations are less productive in favorable cropping environments but tend to greater general persistency, and may be higher-yielding even in the short term under severe stress conditions, e.g., drought (Annicchiarico and Pagnotta, 2012). An important example of genetic resources in red clover is the Swiss Mattenkleee landraces, which form a genetically distinct group from Swiss wild populations according to amplified fragment length polymorphism (AFLP) analysis, with important implications for conservation and breeding (Kölliker *et al.*, 2003). In an extensive survey of 12 red clover populations, genetic diversity was found to be particularly high in Swiss wild clover populations and Swiss Mattenkleee landraces (Herrmann *et al.*, 2005). Extensive genetic diversity studies have also been carried out in other parts of the world including the United States (Taylor, 2008) and Chile (Campos-de-Quiroz and Ortega-Klose, 2001), leading to the development of improved red clover cultivars.

As in white clover, though at a less intense level, several programs of interspecific hybridization between red clover and related species have been carried out (Abberton, 2007). The main emphasis has been on increasing longevity through crosses with more persistent clover species, particularly zig-zag clover (*T. medium*), a species which occurs as a polyploid series with chromosome numbers of $2n = 48-126$ (Williams and Nichols, 2011). However, these hybrids have no commercial impact to date.

Studies of molecular diversity are increasingly performed on genetic resources of red clover, alfalfa and white clover with different objectives, such as exploring phylogenetical or evolutionary relationships (e.g., Muller *et al.*, 2006), investigating the genetic structure of populations (e.g., Crochemore *et al.*, 1996), assessing innovative procedures for variety distinctness (e.g., George *et al.*, 2006), identifying genetically-contrasting material for inclusion in semi-hybrids or in narrowly-based synthetic varieties (e.g., Scotti *et al.*, 2011), and predicting the patterns of variation between populations that could be expected for morphophysiological traits. The latter aim has great practical importance for establishing core collections or choosing subsets of populations for evaluation activities, but requires fairly good consistency between molecular and morphophysiological information. Results from various studies, e.g., Greene *et al.* (2004) and Pagnotta *et al.* (2011) for red clover, Crochemore *et al.* (1998) for alfalfa, and Kölliker *et al.* (2001b) for white clover, substantially challenge this assumption - although more conclusive results are awaited from studies based on high numbers of markers (such as those allowed for by next-generation sequencing techniques). Information is lacking on the ability of molecular studies to predict the level of intra-population

morphophysiological diversity featuring different populations, or to predict combining ability among populations.

IV. TARGET TRAITS AND RELEVANT GENETIC VARIATION

Traits and their rate of improvement are the defining objectives of plant breeding systems. Analysis of trait improvements over time demonstrates the value proposition of forage breeding (Table 1; Bouton, 2007), with notable cases of forage legume improvement giving rise to improved animal performance (Bouton *et al.*, 2005).

In forage legumes, agronomic traits such as forage yield and persistence, compatibility with grasses, tolerance to abiotic and biotic stresses, grazing tolerance, and forage quality are of value to farmers and the seed industry. Seed yield *per se* is valued by the herbage seed industry as the economic unit within their business, and indirectly by farmers in that seed is necessary at an economic price for grassland establishment. The balance between reproductive and vegetative growth is an ongoing optimization challenge for breeding programs. Similarly, the balance between forage yield potential and persistence is a longstanding challenge (Jahufer *et al.*, 2002).

Prioritization of traits in setting breeding objectives is a challenge for breeders, given the negative genetic correlations among some favored trait combinations, and the varying value that different farm systems or industries place on different traits. For example, in marginal environments the value of abiotic stress tolerance is paramount (Smith and Fennessy, 2011). In more temperate regions with reliable rainfall or irrigation, greater focus may be placed on forage yield and quality traits, whereas plant survival can be important under frequent defoliation (Annicchiarico, 2007b). There is also interest in traits enabling forage legumes to better integrate into grazing systems, or enhancing their compatibility with forage grasses in mixtures (Humphries, 2012).

Trait performance benchmarking is a useful tool for improved grower certainty in selecting appropriate varieties. It is essential that the benchmarking conditions are relevant to farm systems, and that the signals to breeders are stable. The challenge this provides for improving white clover is illustrated by the management by leaf size interactions (Gilliland *et al.*, 2009). Varying environmental conditions and use of different benchmarking standards within and among countries creates further challenges (Parsons *et al.*, 2011) and warrant further research to aid breeders in trait prioritization.

A. Forage Yield

Forage yield of vegetative matter is of primary interest in all forages legumes whether harvested mechanically or by grazing. In natural populations, there is a balancing selection for reproductive and vegetative growth (Sawada, 1999), which plant breeders seek to alter. In many cases, newly-sown swards tend to revert to a background ecotype, or show genetic shifts in traits

as the populations adapt to specific pressures (Lane *et al.*, 2000; Göransson *et al.*, 2012).

Local adaptation is critical for optimal yield expression (Oliveira *et al.*, 2013). One way of achieving improvement in locally adapted forage legume germplasm has been to hybridize ecotype germplasm with elite populations (Bouton *et al.*, 2005), to capitalize on specific-adaptation effects that frequently feature landraces and natural populations (see Section III).

Improving forage yield begins with definition of target environments and systems through multi-site evaluation (Annicchiarico *et al.*, 2011). A decision on the target width of adaptation is necessary and influences the choice of genetic resources and selection environments. For example, alfalfa breeding programs in the United States generate varieties with different autumn dormancy whose winter hardiness matches the requirements of their specific target region (McKenzie *et al.*, 1988). Greater yield gains have arisen from alfalfa breeding for specific adaptation to moisture-favorable or drought-prone conditions (Annicchiarico, 2007c). White clover has largely been bred for specific adaptation to contrasting exploitation regimes, i.e., mown or lax rotational grazing or continuous grazing (Williams, 1987b).

The adoption of innovative or improved selection schemes, which is particularly relevant to forage yield improvement, will be discussed in Section VI, along with opportunities for enhancing breeding schemes through incorporation of marker information and improved phenotyping.

1. Alfalfa

Despite extensive variation within populations (Julier *et al.*, 2000; Annicchiarico, 2006a), improving the yield potential of alfalfa in favorable cropping environments has proven difficult, and remains a central challenge in forage breeding (see also Section II). Tetrasomic inheritance and high rate of non-additive genetic variance arising from gene interaction, severe inbreeding depression, and large genotype \times environment interactions, increase the complexity of this task (Bingham *et al.*, 1994; Veronesi *et al.*, 2010; Annicchiarico *et al.*, 2010). Trial design to assess yield differences requires close scrutiny, given the fine differences in yield potential that may exist (Casler and Undersander, 2000).

Alfalfa long-term yield in favorable cropping environments, especially under frequent mowing, is strictly associated with higher amounts of starch and soluble proteins in the root and the crown due to higher concentrations and greater size of organs (Annicchiarico, 2007b; Annicchiarico *et al.*, 2013). Indirect selection based on crown diameter proved valuable in the study by Bakheit *et al.* (2011), while Kimbeng and Bingham (1998) found it less useful than crown health. Selection for greater root biomass resulted in higher forage yields (Pederson *et al.*, 1984), while work by Lamb *et al.* (2000) suggested the particular value in this context of greater proportion of lateral or fibrous roots. However, direct selection for aerial biomass under frequent mowing may prove more cost-efficient than

indirect selection for root traits (Rotili *et al.*, 1994), unless reliable and relatively inexpensive methods could be devised for root phenotyping. The difficulty of selecting for root size could be alleviated by estimations based on root diameter or electrical capacity (Kendall *et al.*, 1982) and, possibly, by selection under hydroponic or *in vitro* conditions (as attempted in other crops: e.g., Izumi *et al.*, 1997). General symbiotic aptitude as measured by higher nodule biomass per plant is another below-ground indirect selection criterion for improving forage yield traits which, however, was reportedly less efficient than direct selection for aerial biomass (Scotti *et al.*, 2007).

Severe plant mortality occurring in cultivated stands over the crop cycle tends to eliminate less vigorous and adapted genotypes (Veronesi and Lorenzetti, 1983; Hotchkiss *et al.*, 1996), suggesting that mass selection of surviving plants from dense stands which are carefully managed to minimize the environmental heterogeneity (Annicchiarico, 2004) may provide an inexpensive means of improving populations and breeding stocks for specific growing conditions. On the other hand, genotype selection under spaced planting (i.e., in the absence of intraspecific competition), although frequently adopted in forage breeding (Poehlman and Sleeper, 1995; Posselt, 2010), was predicted to provide only about 60% of the forage yield gains provided by selection under dense planting (Annicchiarico, 2006b). This is caused by the only moderate genetic correlation for genotype yield responses across these conditions (Table 4; this agrees with phenotypic correlation values reported in other studies, e.g., Riday and Brummer, 2004). Such inconsistency of genotype responses can be attributed to the different yield components which affect primarily the yield responses in the two conditions, i.e., tall canopy under dense planting, and production of tillers under spaced planting (Rotili and Zannone, 1975). Forage yield under spaced planting could be indirectly selected for on the basis of plant height, which is little affected by genotype ×

density interaction. However, direct selection for forage yield under relatively dense planting (e.g., 10–12 cm spacing between and within rows) is quite feasible, is compatible with other scientific objectives (e.g., studies of biodiversity, genetic structure, or estimation of genetic parameters), and can be improved by using grid selection and spatial adjustment procedures (Annicchiarico, 2004).

Despite their practical importance for selection progress, published estimates of narrow-sense heritability for alfalfa forage yield are very rare. Reported values range from 0.14 (Pecetti and Piano, 2005) to 0.30 (Riday and Brummer, 2005). Efforts to decipher the genetic basis of variation for yield are reported from researchers using biparental mapping populations. These have led to the discovery of quantitative trait loci (QTL), and associated candidate gene polymorphism with yield potential (Robins *et al.*, 2007a). A candidate gene, CONSTANS-like, has also been associated with stem height, a determinant of yield (Herrmann *et al.*, 2010).

The open-pollinated nature of alfalfa, risk of inbreeding depression and quantitative nature of yield potential, indicate that marker effects need to be established and utilized in diverse breeding populations if they are to be of value in genetic improvement. This strategy can work using low density marker systems (Maureira-Butler *et al.*, 2007; Li *et al.*, 2011) in recently combined populations and cases where moderate levels of linkage disequilibrium are detected. This offers the advantage of not having to conduct QTL discovery in biparental populations, and is indicative that genomic selection strategies may be useful in improving yield (see Section VI).

Yield heterosis is a longstanding interest in alfalfa breeding, with studies confirming heterotic effects can be detected, additive effects through general combining ability as the primary basis (Maureira *et al.*, 2004; Bhandari *et al.*, 2007; Madril *et al.*, 2008; Al Lawati *et al.*, 2010). Forage yield is more sensitive to

TABLE 4
Estimated genetic correlation for dry matter yield of alfalfa, white clover and red clover material across selection (or phenotyping) and target conditions

Selection	Target	Species	Material	Correlation	Source
Spaced plant	Dense planting	Alfalfa	16 genotypes	0.71	Annicchiarico (2006b)
Spaced plant	Dense planting	White clover	12 genotypes	0.60	Annicchiarico and Piano (2000)
Pure stand	Association with grasses	White clover	165 genotypes	0.67	Annicchiarico (2003)
Pure stand	Association with grasses	White clover	47 genotypes	0.64 ^a	Rowe and Brink (1993)
Favorable	Drought-prone	Alfalfa	17 populations	-0.34	Annicchiarico and Piano (2005) ^b
Favorable	Drought-prone	Alfalfa	12 populations	0.09	Annicchiarico <i>et al.</i> (2011) ^b
Favorable	Drought-prone	Alfalfa	20 populations	-0.25	Annicchiarico <i>et al.</i> (2012)
Favorable	Drought-prone	Alfalfa	90 half-sib families	0.66	Annicchiarico, unpublished data
Favorable	Drought-prone	Red clover	19 populations	0.34	Annicchiarico and Pagnotta (2012) ^b
Non-saline	Saline	Alfalfa	12 populations	0.71	Annicchiarico <i>et al.</i> (2011) ^b
Mowing	Sheep grazing	White clover	20 populations	0.05	Annicchiarico (2012) ^b

^aAverage correlation for two association conditions.

^bComputed from reported yield data.

genetic diversity in this tetraploid species than in diploids, because of complementary gene interactions involving favorable alleles with additive effects in linkage blocks (Bingham *et al.*, 1994). Possible innovative variety types designed to exploit heterosis and non-additive genetic effects are discussed in Section VI.

2. White clover

Commonly used in temperate grazing systems, white clover displays a remarkable plasticity of yield and morphology in response to differential environmental signals, including defoliation management (Evans *et al.*, 1998; Gilliland *et al.*, 2009). There is extensive variation for yield potential in diverse and elite white clover populations (Aasmo Finne *et al.*, 2000a; Jahufer *et al.*, 2013), even within full-sib progenies. As an indirect measure of yield potential, leaf size is a useful, readily measured trait (Caradus and Chapman, 1998), with recent research demonstrating the ability to automate estimation of clover yield using a combination of digital imaging and point analysis (Chen *et al.*, 2010).

Yield responses of white clover genotypes or populations may largely interact with cropping environments (Jahufer *et al.*, 2013). They can also be inconsistent across contrasting conditions of plant density, type of grass companion and exploitation regime (Table 4), with important implications on breeding strategies.

A general trade-off exists between yield potential and plant persistence, especially under grazing (Williams, 1987b; Caradus and Chapman, 1996). The trade-off derives from the inverse genetic correlation between stolon and leaf biomass, and the strict positive association of persistence with stolon production and of yield potential with leaf and petiole size (Annicchiarico *et al.*, 1999). Thus, persistent types are generally identified as smaller leaved, lower yielding and highly stoloniferous (Williams, 1987b). The relationship of stolon density with long-term yield allows for effective early indirect selection for the latter trait, with considerable time and cost savings (Annicchiarico, 1997). The negative correlation between yield and persistence has been partially overcome in some notable cases, enabling parallel improvement of both traits (Caradus *et al.*, 1997).

Within a given environment, the narrow-sense heritability of forage yield components is in the range 0.40-0.70 (Annicchiarico *et al.*, 1999 and earlier studies summarized there). Its value for forage yield, however, ranged from about 0.50 (Annicchiarico *et al.*, 1999) to about nil (Woodfield and Caradus, 1990); this latter value contrasts with breeding gains reported in Section II.

Local adaptation is important, as shown by the poor performance of many foreign cultivars in the study by Pederson *et al.* (1999). There are notable cases, however, in which foreign cultivars have made excellent parents when combined with local ecotypes (Bouton *et al.*, 2005). At the extremes, local adaptation of ecotypes can outperform bred material (Annicchiarico, 1993; Brink *et al.*, 1999).

The use of wide hybridization and trait introgression has been pursued to improve forage yield potential and adaptation, with indications of yield potential improvements (Marshall *et al.*, 2002a; Marshall *et al.*, 2005). Evaluation under grazing has also shown this potential (Marshall *et al.*, 2002b), providing some evidence that interspecific hybrids with *T. nigrescens* are superior under grazing. Work done with mechanical defoliation has shown that differences among lines may not persist over time (Marshall *et al.*, 2003).

A systematic assessment of phylogeny in the *Trifolium* genus (Ellison *et al.*, 2006) has enabled further utilization of wide hybrids (Williams *et al.*, 2011) and provides experimental evidence for identifying the progenitor species of allotetraploid white clover (Williams *et al.*, 2012). This has implications for introgression breeding influencing the agronomic adaptation of white clover to a range of ecophysiological and pasture management conditions.

Just like alfalfa, also white clover undergoes severe plant mortality of less vigorous individuals in cultivated stands, although a trade-off between competitive ability and reproductive ability can act as a buffer against rapid change of genotype frequencies (Annicchiarico and Piano, 1997). Genotype yield responses across spaced and dense planting conditions exhibited only fair genetic correlation (Table 4), owing to the greater importance as a yield component of stolon production in the former condition and leaf size in the latter (Annicchiarico and Piano, 2000). Spaced plant selection was predicted to provide about 60% of the forage yield gains provided by dense plant selection obtained by close transplanting of a few clonal cuttings (Annicchiarico and Piano, 2000).

3. Red clover

Red clover, more commonly used in mechanically harvested forage systems, shows extensive genetic variation for yield (Rosso and Pagano, 2005; Hejduk and Knot, 2010; Annicchiarico and Pagnotta, 2012; Vymyslicky *et al.*, 2012), including clines of adaptation for heat and cold tolerance as they relate to yield and persistence. Studies using molecular markers have also revealed extensive genetic diversity (Dias *et al.*, 2008; Vymyslicky *et al.*, 2012), with rapid genetic change of broad populations grown in contrasting environments (Collins *et al.*, 2012).

In any species, establishment is necessary for agronomic traits to be expressed. Frost seeding of red clover is used to renew degraded pastures in cold environments, but seed establishment in these conditions has low heritability (Riday, 2008). Seedling height is a more heritable character that may be of greater use in selecting for establishment vigor. Yield characterization in red clover spaced plant nurseries can be accelerated by the use of visual scores of biomass, which have been shown to be robust across evaluators (Riday, 2009). Also red clover showed only moderate correlation for genotype yield responses across dense and spaced planting (Real *et al.*, 2000).

Persistence is a longstanding challenge in red clover. Swiss Mattenkleee ecotypes (Boller *et al.*, 2004) have been a source of persistence in breeding programs. Wild populations feature higher levels of persistence than landraces (Annicchiarico and Pagnotta, 2012), but their exploitation as a genetic resource has been modest because of concurrently lower forage yield potential (Boller *et al.*, 2010a). Red clover persistence is also markedly affected by tolerance to diseases (Taylor, 2008; Boller *et al.*, 2010a). Genetic analyses including QTL discovery have contributed to understanding of the trait, with QTL for stem length, seed yield and persistence all co-located (Herrmann *et al.*, 2008). An independent study identified QTL for plant persistence on groups 3 and 6 (Klimenko *et al.*, 2010). Narrow-sense heritability estimates for forage yield traits ranged from moderate to low (Taylor and Smith, 1979).

Some exploration of the potential of interspecific hybrids has been reported, with progress made towards improving persistence in backcross generations of crosses with *T. medium* (Isobe *et al.*, 2002). These insights into the genetic control of yield and persistence offer continued opportunities to improve this valuable temperate forage legume.

B. Plant Competition and Compatibility with Grasses

This subsection will focus on implications for breeding of interspecific competition in: (i) legume-grass mixed cropping, with the aim to obtain balanced, long-lasting mixtures; and (ii) against weeds, to reduce or eliminate chemical weed control.

As anticipated, mixed cropping with forage grasses is the usual practice for white clover and is frequent for alfalfa and red clover. Selecting legume genotypes in pure stand is simpler and less expensive than selecting in mixed stand, but a crucial question for breeders is whether this selection environment can effectively be used also for selecting varieties suitable for mixed cropping. There are several possible grass companion species (perennial ryegrass, *Lolium perenne*; hybrid ryegrass, *L. hybridum*; Italian ryegrass, *L. multiflorum*; tall fescue, *Festuca arundinacea*; cocksfoot, *Dactylis glomerata*; timothy, *Phleum pratense*; brome grass, *Bromus* spp.; bermudagrass, *Cynodon dactylon*; etc.), and many varieties within each species. The question of whether the suitability for mixed cropping of a legume variety tends to be a general characteristic, or varies depending on the grass companion (thereby introducing further complications in the selection work), is critical in defining breeding strategy. Most available indications relate to studies on white clover, owing to the special importance of mixed cropping for this species.

Associating white clover with a vigorous grass variety under moderate levels of nitrogen fertilization is often adopted to reach acceptable forage yield levels, but imposes severe competition on the clover as reflected by decreasing proportion and poor persistence of the clover in the stand (Harris, 1987). In general, white clover has lower ability to compete for light and nutrients than most grass companions (Haynes, 1980), and its selection for competitive ability against associated grasses has proved to

be a valuable strategy to obtain compatible clover-grass mixtures (Annicchiarico and Proietti, 2010). A compatible mixture maintains a clover content large enough to optimize the benefits of its nitrogen fixation and superior forage value within a high-yielding grass background (Rhodes *et al.*, 1994). In this respect, the target clover content in the association has been estimated around 30% on an annual dry matter basis (Thomas, 1992). A typical feature of compatible mixtures relative to non-compatible ones is the occurrence of higher clover yield accompanied by similar grass yield (Evans *et al.*, 1989; Annicchiarico and Piano, 1994), in agreement with the general observation that the yield efficiency of a mixture is mainly determined by the performance of its weaker partner (Harper, 1977). The absence of grass yield decrease in compatible mixtures can be explained by the fact that the negative effect of increased competition exerted by the clover is counterbalanced by the greater amount of biologically-fixed nitrogen available for the grass growth from decay of clover leaf and stolon (Chapman *et al.*, 1996).

Genetic correlations, averaging about 0.65, indicate moderate consistency between white clover genotype yield responses in pure vs. mixed stand selection environments (Table 4). Taking account of broad-sense heritability values in each environment, these studies suggested 12 to 43% greater efficiency for selection of medium-leaved and large-leaved genotypes in mixed stands relative to pure stands (Rowe and Brink, 1993), and over two-fold greater efficiency for selection of large-leaved genotypes in mixed stands (Annicchiarico, 2003). Furthermore, the inclusion in the latter study of small-leaved material increased the genetic correlation across conditions and decreased to 43% the relative advantage of selection in mixed stand, owing to the importance of taller canopy (as conferred by larger leaves) for clover competitive ability (Harris, 1987) and its positive relationship with yield in pure stand. When selecting within a given leaf size class (as it is usually the case), clover competitive ability is also associated with other traits, such as stolon production and petiole plasticity (i.e., the ability to produce petiole elongation in response to grass competition through a physiologically-mediated response to shading: Robin *et al.*, 1992) (Annicchiarico, 2003). These traits contribute to enhanced precision foraging, i.e., the ability to make fine-scale exploitation of light and nutrients from undepleted zones, which is fundamental for the competitive success of weaker partners in associations (Campbell *et al.*, 1991). Other traits are known to also contribute to white clover competitive ability in other contexts, e.g., winter hardiness and rapid spring growth in cold-prone regions (Rhodes *et al.*, 1994). The knowledge of traits associated with competitive ability can increase the efficiency of pure stand selection targeted to mixed stand conditions (Annicchiarico, 2003).

The consistency of white clover genotype yield responses across several association conditions was thoroughly investigated by Annicchiarico and Piano (1994) for clover material grown in pure stand and in binary mixture with each of eight grass varieties belonging to four different species. Clover genotype \times evaluation environment interaction took place both

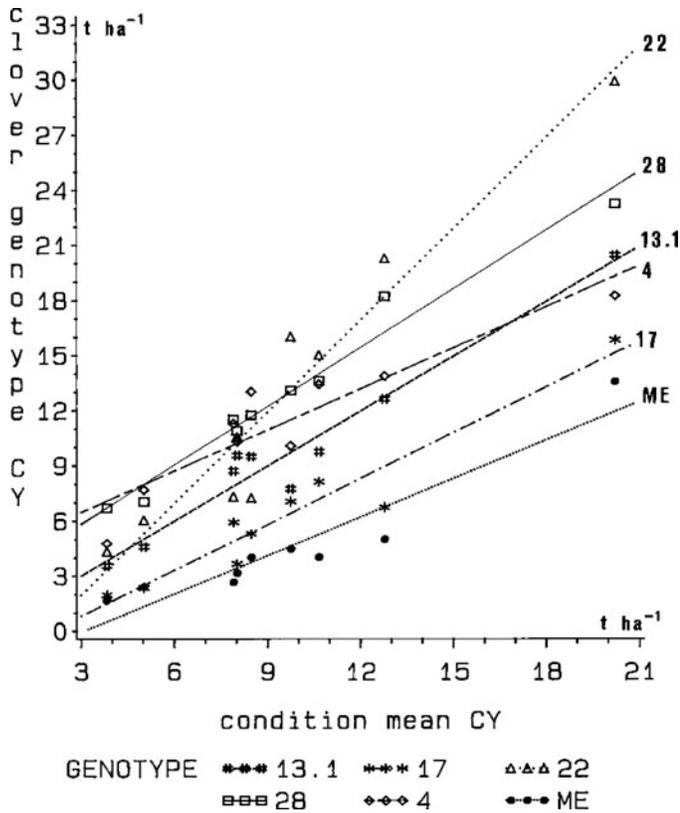


FIG. 3. Regression of two-year clover dry matter yield (CY) as a function of mean clover yield in the growing condition, for six white clover genotypes grown in the conditions of pure stand and of binary mixture with each of eight grass varieties belonging to four species (reprinted from: Annicchiarico and Piano, 1994).

between monoculture and average of mixtures, and among mixtures. However, it was simply interpretable in terms of variation of genotype regressions as a function of increasing grass competition as measured by decreasing clover mean yield (Figure 3). Significant rank inversions occurred between nil or low interspecific competition (pure stand or mixed stand with the least competing grass variety) and severe interspecific competition (mixed stand with highly competing grass companions). This pattern of response could explain why clover genotype \times evaluation environment interaction did not emerge consistently in earlier experiments summarized in Annicchiarico and Piano (1994), and suggests that it is the level of target competitive stress rather than the target grass species or grass variety that needs to be defined in a breeding program. The extent of competitive stress depends on genetic features of the grass companion, such as its intrinsic yielding ability (Piano and Annicchiarico, 1995), morphology of leaves and roots, and seasonal growth pattern (Collins and Rhodes, 1990), and by environmental and agronomic factors, especially nitrogen fertilization and mode and frequency of exploitation (Frame and Newbould, 1986).

Compatibility effects leading to higher total yield of specific white clover-grass pairs were reported for white clover geno-

types associated with grass genotypes (Turkington, 1989a) or grass populations (Evans *et al.*, 1989; Lüscher *et al.*, 1992) with which they had previously coexisted in a natural grassland. However, the exploitation for breeding of these specific compatibility effects has been hampered by: (i) their partly transient nature (Turkington, 1989b); (ii) the practical difficulty and the limited economic value of marketing specific pairs of grass and clover varieties. Broader adaptation is generally required to achieve sufficient market size.

The relationship of leaf size with competitive ability sets a limit to white clover-grass mixtures that can be compatible. Even highly-competitive medium-leaved clover varieties are unlikely to be compatible with vigorous grass companions, especially in mown, environmentally-favorable environments. Actually, even large-leaved clover material may be severely outcompeted by very vigorous cocksfoot varieties in these conditions (Harris, 1987; Annicchiarico and Proietti, 2010).

Unlike white clover, alfalfa and red clover may exhibit competitive advantage over a vigorous grass companion, particularly under cropping conditions well-suited to the legume component (Chamblee and Collins, 1988). There is very limited information on the consistency of alfalfa or red clover genotypes across pure stand and mixed stand selection environments. Work on alfalfa (Zannone *et al.*, 1986) highlighted: (i) the major impact of plant stature on the competitive ability of alfalfa populations (as expected for erect, dominant species in favorable environments: Campbell *et al.*, 1991); (ii) the consistency of alfalfa genotype yields across pure stand and mixed stand conditions, which derives from the crucial importance of plant stature also for high yielding ability in monoculture; and (iii) the advantage for mixed cropping of choosing legume-grass pairs that are top-yielding in the pure stand assessments for the respective species, provided that their competitive abilities (predictable from their yields in pure stand) are not too different. These results emphasize the importance of improving the competitive ability of the weaker partner species in the target conditions and, if this is not sufficient, of breeding the dominant species for mixed cropping by selecting for lower competitive ability. In this respect, ongoing work in France aims to verify whether lower stem height, more branching, and good regrowth dynamics after mowing may provide acceptable yielding ability in pure stand along with greater compatibility with grass companions (Maamouri *et al.*, 2014). Crop establishment procedures (e.g., sowing date and density; seeding pattern) and crop management (e.g., fertilization; mowing height and frequency) remain important for driving and maintaining a desirable balance of legume and grass components under cultivation (Frame and Newbould, 1986; Chamblee and Collins, 1988).

Endophyte presence in the companion grass is another major factor in the competitive balance between grass and legumes clover, which can suppress clover yields in some circumstances (Hoveland *et al.*, 1999).

The ability to compete with weeds has special importance for legume monocultures (which are more subjected to weed

invasion than legume-grass mixtures) and organic systems (which do not allow any chemical weed control). Its genetic variation in perennial legume species has not been thoroughly investigated. A recent study on alfalfa (Annicchiarico and Pecetti, 2010) highlighted the strict relationship of competitive ability in the presence of severe weed invasion with forage yielding ability in the absence of weeds (obtained through chemical weed control), in agreement with the expected relationship of both traits with taller stature and higher relative growth rate. Thus, selection for high aerial biomass in the absence of weeds (as ordinarily done) would effectively select also for high competitive ability against weeds.

Chemical control of weeds in mixed swards can have a negative impact on forage legume survival and yield. This has prompted interest in selection for herbicide tolerance in these species, and of herbicides that are less damaging to clover (McCurdy *et al.*, 2013).

C. Tolerance to Abiotic Stresses

1. Current and expected future importance of major stresses

Greater tolerance to abiotic stress is of inherent value to forage legume growth and persistence, and can improve the sustainability of these crops in agricultural systems. In rainfed systems, or under limited economic access to water in irrigated systems, greater water use efficiency (WUE) or other mechanisms of drought tolerance have special value. These characteristics will increase their importance in the context of climate change, owing to the predicted increase of drought stress (arising from higher temperatures and less rainfall) and reduction of available irrigation water (driven by increased demand for urban water and drying water tables) (Parry *et al.*, 2007). Tolerance to salinity is likely to increase in importance, particularly because of the expected increased irrigation with saline water (Leone and Steduto, 1998).

Cold tolerance is likely to remain important in two major contexts, i.e.: (i) the progressive introduction in northern latitudes of winter-active varieties, which is favored by climate change but implies selection for local adaptation of cold-susceptible genetic resources; and (ii) the greater climatic variation which is also expected as a consequence of climate change (Parry *et al.*, 2007) which would imply greater frequency of early or late winter frosts on non-hardened or dehardened plants. Increasing costs of mineral fertilizers and the possible expansion of cultivation onto marginal or less productive land may draw increased attention to phosphorous uptake and utilization efficiency.

Forage perennial legumes exhibit a range of adaptation to drought (e.g., Pang *et al.*, 2011) and other abiotic stresses, offering insights into characters to improve in breeding programs, in addition to physiological mechanisms.

2. Drought tolerance

Drought tolerance for perennial legumes could be defined as the ability to survive and produce forage after various dry seasons. Soils with low water holding capacity can exacerbate

the negative effects of drought spells. In alfalfa, drought tolerance has not been a major breeding target until recently, and the extent of genetic variation for this trait and its contributing mechanisms have not been thoroughly elucidated. The presence of large variation is supported by the occurrence of wide genotype \times environment interaction of cross-over type (i.e., implying significant changes in population ranks) for forage yield across drought-prone and moisture-favorable agricultural environments, observed not only in a relatively large region such as the western Mediterranean basin (Annicchiarico *et al.*, 2011) but also in a relatively small region such as northern Italy (Annicchiarico and Piano, 2005). Germplasm adaptive responses in these studies were clearly associated with stress levels of landrace collecting environments and variety selection environments. Results from various studies summarized in Table 4 indicate, in most cases, about nil genetic correlation for population yield responses across drought stress and moisture-favorable conditions. This finding can be accounted for by the different and partly incompatible morphophysiological traits associated with optimal plant adaptation to these conditions (Annicchiarico, 2007b; Annicchiarico *et al.*, 2013). Accordingly, greater breeding progress would arise from specific selection for stress or favorable environments rather than selection for wide adaptation, as confirmed experimentally for northern Italy (Annicchiarico, 2007c). Results by Ray *et al.* (1999a; 1999b) suggest that breeding for wide adaptation could rely on selection for higher WUE as tracked by carbon isotope discrimination, which is heritable and can be selected in tandem with yield for improved performance under irrigation. Differences in stomatal conductance have been shown to account for increased WUE in *falcata* germplasm, and offer an opportunity to introgress WUE into genetically broader germplasm (Ray *et al.*, 2004).

Higher levels in subterranean organs of osmotically-active nitrogen compounds and water soluble carbohydrates can contribute to alfalfa drought resistance (Pembleton *et al.*, 2010; Annicchiarico *et al.*, 2013). Work by Kang *et al.* (2011) suggests that drought-tolerant and drought-susceptible germplasm may share similar physiological mechanisms of stress response, the main differences being quantitative (e.g., for decrease of stomatal conductance under stress, or production of osmolytes and antioxidants). Use of trehalose in transgenic plants has also been shown to confer tolerance to drought stress (Suárez *et al.*, 2009). Morphological adaptations to drought include prostrate growth habit and rhizomatous roots in germplasm collections (Prosperi *et al.*, 2006), traits also associated with grazing tolerance. Lateral roots are favored in dry environments, but the extent of genetic variation for this trait is somewhat controversial (Lamb *et al.*, 2000; Odorizzi *et al.*, 2008). Intrinsically higher stem number (as shown in stress or favorable environments) was a feature of drought tolerant germplasm in different studies (Annicchiarico, 2007b; Annicchiarico *et al.*, 2013). Lower leaf wilting and leaf senescence under stress are inexpensive morphological traits which could be used in early screening for drought tolerance (Kang *et al.*, 2011; Annicchiarico *et al.*, 2013).

White clover, featuring a shallow root system and poor stomatal regulation (Hart, 1987), is recognized as being relatively poorly adapted to drought. This is further complicated in mixed swards, where ryegrass can increase water use efficiency, putting further pressure on clover (Lucero *et al.*, 2000). Various studies (e.g., Barbour *et al.*, 1996; Brink and Pederson, 1998) have reported forage yield variation among white clover populations to flatten out passing from moisture-favorable to drought stress conditions with no genotype \times environment interaction of cross-over type, suggesting that drought tolerance improvement would be hindered by modest genetic variation. However, field selection has led to improved material identified within full-sib families (Jahufer *et al.*, 1999). This finding, applied as an *in situ* selection strategy, has resulted in the development of relatively drought tolerant cultivars adapted for summer moisture stress with persistence and broad adaptation (Ayres *et al.*, 2007).

White clover root characteristics are amenable to selection, although exhibit lower heritability than shoot traits (Caradus and Woodfield, 1990). Driven primarily by additive variance, it has been shown possible to select for fewer larger roots and larger taproot diameter (Woodfield and Caradus, 1990). Assessment of divergent selections in the field showed increased root:shoot ratio, root diameter and root numbers all be associated with increased yield in moisture-limited and well-watered conditions (Caradus and Woodfield, 1998). Indirect selection for stolon root development based on stolon diameter increased root dry matter, although this did not led to improved drought tolerance in the test conditions (Annicchiarico and Piano, 2004).

Recent work to elucidate the physiological and metabolic effects on abiotic stress tolerance in white clover has identified quercetin, a flavonoid compound, as a possible compound of interest for plant breeders (Hofmann and Jahufer, 2011). Investigation in relation to drought response shows a drought protective effect associated with the compound, which is effective across environments (Ballizany *et al.*, 2012).

Ecotype germplasm has been used as a genetic resource to improve white clover drought tolerance in different countries (USA; New Zealand; Uruguay). Utilization of the secondary and tertiary gene pool may also offer morphological and other adaptations to drought tolerance (Marshall *et al.*, 2001). Recent evidence on the putative progenitor species and related germplasm that can be introgressed (Williams *et al.*, 2011; Williams *et al.*, 2012) suggests there are opportunities for adaptive introgression of drought tolerance into white clover.

Research and breeding efforts on red clover drought tolerance have been very limited, despite its recognized importance as a breeding objective (Ravagnani *et al.*, 2012) and its contribution to longevity of the crop (Abberton and Marshall, 2005). Comparative studies on the dry matter yield of different forage legume species in response to drought have shown red clover to be more sensitive than alfalfa (Peterson *et al.*, 1992).

Red clover exhibited genotype \times environment interaction of cross-over type and low genetic correlation for population yield values across drought-prone and moisture-favorable en-

vironments (Table 4), in a study which highlighted the great value as a genetic resource for drought-prone regions of wild populations that evolved in rainfed Mediterranean environments (Annicchiarico and Pagnotta, 2012). Work in progress is identifying other drought-tolerant germplasm sources and analyzing differences in gene expression under drought between drought-tolerant and drought-sensitive material (Yates *et al.*, in preparation).

3. Cold tolerance

Cold tolerance has been a major breeding objective for perennial forage legumes in more continental and high latitude areas. In alfalfa, the primary adaptive traits are variation in fall dormancy, and freezing tolerance, both of which have been improved by breeding. While there is evidence that fall dormancy and freezing tolerance are correlated in some germplasm (Cunningham *et al.*, 1998; Cunningham *et al.*, 2001), they have also been shown to be independently inherited (Brummer *et al.*, 2000). The independence of these two traits has been confirmed in mapping studies (Brouwer *et al.*, 2000), with some fall dormancy and winter injury effects detected in both similar and unlinked positions in the genome. Hybrids among populations of differing fall dormancy may offer a means to develop populations suited to environments with intermittent freezing conditions (Bhandari *et al.*, 2007).

Efforts to develop efficient trait screening assays for alfalfa have focused on plant material where seedlings and cuttings have been shown to be effective (Brouwer *et al.*, 1998), and development of controlled environment methods for freezing tolerance (Castonguay *et al.*, 2009).

There is heritable variation for alfalfa cold adaptation, especially noted in ecotype and feral populations (Basafa and Taherian, 2009; Bagavathiannan *et al.*, 2010). Recurrent divergent selection in breeding populations have created distinct populations, where genetic analysis has shown that variation at dehydrin loci are associated with freezing tolerance (Rémus-Borel *et al.*, 2010). A simple length polymorphism assay has been developed for use in breeding programs, and has shown a specific variant to be associated with freezing tolerance (Castonguay *et al.*, 2012). Higher expression of dehydrin genes has been shown to be related to higher cold resistance (Dubé *et al.*, 2013). Two recent studies using linkage mapping on *M. truncatula* have identified a QTL region including substantial effects in chromosome 6 relating to freezing tolerance, with comparative genomics tools in the high density map showing co-linearity with a locus on *Pisum* group 6 (Avia *et al.*, 2013; Tayeh *et al.*, 2013).

White clover offers a remarkable range of adaptation for cold tolerance, provided hardening and de-hardening periods are ensured. Substantial adaptation changes at the population level have been shown to be rapid, happening within three years of establishment (Helgadóttir *et al.*, 2001). Like most cold tolerance mechanisms, soluble sugar, protein and amino acids including proline are implicated (Svenning *et al.*, 1997; Annicchiarico

et al., 2001; Frankow-Lindberg, 2001), in addition to lipid saturation in stolons (Collins *et al.*, 2002).

White clover is a broadly adapted species, with populations growing near polar latitudes exhibiting high levels of cold tolerance (LT₅₀ of -16°C), and retaining yield potential in some cases matching bred material (Svenning *et al.*, 2001). Selection has been shown to be effective to improve both cold tolerance and low temperature growth (Rhodes *et al.*, 1994), the latter trait of particular value in year round grazing systems supplying winter feed that often defines the carrying capacity of a farm. Response to tandem selection for yield and winter hardiness has been successful in improving material for Scandinavian regions (Aasmo Finne *et al.*, 2000b; Helgadóttir *et al.*, 2008) and more temperate zones (Lüscher *et al.*, 2001). Artificial frosting environments for selection have also been developed (Collins and Rhodes, 1995; Caradus and Christie, 1998). Greater cold tolerance was reportedly associated with stolon content of water-soluble and total non-structural carbohydrates (Collins and Rhodes, 1995; Frankow-Lindberg, 2001) and soluble proteins (Annicchiarico *et al.*, 2001).

Cold tolerance contributes to red clover yield and persistence in northern latitudes (Klimenko *et al.*, 2010; Ravagnani *et al.*, 2012), but its basis and genetic variation have been less investigated than in alfalfa and white clover. Red clover is not considered reliably persistent in cold-winter conditions. Taylor and Quesenberry (1996) reported research on genetic variability for cold hardiness and the possibility of selection for increased cold hardiness. Christie and Choo (1991) evaluated a number of genotypes sorted out by two varieties, reporting greater winter-hardiness for material of the two varieties which did not flower in the sowing year, as well as variation for winter hardiness within both non-flowering and flowering genotype groups. QTL for cold tolerance have recently been identified (Klimenko *et al.*, 2010).

4. Salt tolerance

Soil salinity is a substantial limitation to the zone of adaptation and productive potential of forage legumes. Research to address this challenge is most advanced in alfalfa.

Substantial alfalfa genetic variation for salt tolerance exists which involves various physiological mechanisms, such as Na⁺ exclusion, Na⁺ compartmentation into cell vacuoles, and better retention of K⁺ in plant tissues (Smethurst *et al.*, 2008). *Falcata* material is generally intolerant (Rumbaugh and Pendery, 1990). The Canadian cultivar 'Bridgeview,' Ameristand 801S, and some cultivars from North Africa displayed high tolerance to salt stress (Annicchiarico *et al.*, 2011; Acharya and Steppuhn, 2012; Abd El-Naby *et al.*, 2013).

Field selection in naturally saline soils is hindered by the marked spatial heterogeneity of the stress. The moderate genetic correlation for population yield responses across salt stress and non-stress environments reported in Table 4 requires confirmation, but suggests that field selection in the absence of stress could produce some yield gain also for environments subjected

to this stress. Direct selection at low and moderate levels of salinity has been shown to be effective (Johnson *et al.*, 1992b; Al-Khatib *et al.*, 1993). Assays have been developed including a greenhouse protocol (Peel *et al.*, 2004) and *in vitro* selection (Winicov, 1991; Safarnejad *et al.*, 1996). Selection at multiple growth stages has been shown to be important (Johnson *et al.*, 1992a).

Trait dissection via transcriptome analysis has identified a transcription factor as a candidate gene for alfalfa salt tolerance (Postnikova *et al.*, 2013). QTL for the trait have also been discovered in white clover (Wang *et al.*, 2010), offering some scope for comparative mapping and genomics to identify common factors.

Biotechnology has also been used to test the use of specific transgenes to improve alfalfa salt tolerance (Jin *et al.*, 2010), with examples showing salt tolerance even at 200 mM sodium chloride (Hua *et al.*, 2012).

5. Tolerance to aluminium and acidic soils

Soil acidity and aluminium toxicity are frequently associated and represent further substantial limitations to alfalfa's and clovers' zone of adaptation and yield potential (Bouton and Parrott, 1997). More recently, manganese toxicity has also been shown to significantly reduce alfalfa seedling survival and growth (Hayes *et al.*, 2012a).

Alfalfa cultivar differences have been identified (Grewal and Williams, 2003), and variation within cultivars showing response to selection has been demonstrated in rapid screening systems (Scott *et al.*, 2008; Hayes *et al.*, 2011). Other methods using culture media and soil have been shown to effectively discriminate among tolerant and susceptible types (Khu *et al.*, 2012), and may prove valuable as a screening assay for selection. Alfalfa selection strategies for aluminium tolerance also include QTL discovery aimed at marker-assisted selection (Khu *et al.*, 2013), and transgenesis (Reyno *et al.*, 2013).

Root hairs are severely adversely effected by aluminium in white clover (Care, 1995). White clover is more tolerant to aluminium than alfalfa but offers less scope for improving genetically its tolerance, given the lack of improvement shown under field conditions by populations selected for improved tolerance (Caradus *et al.*, 2001).

6. Low phosphate tolerance

The relatively high levels of P that are required to ensure there is no constraint on forage legume growth is a direct hindrance to farm system efficiency and profitability. Longstanding efforts have been made to improve white clover adaptation to soils with low phosphorous levels. Sustained *ex situ* selection efforts in controlled environments (Caradus, 1994) were shown to be ineffective in the field (Caradus and Dunn, 2000). Physiological dissection of the trait has shown genetic variation for phosphate allocation within the plant, as it relates to stolon vasculature (Lötscher and Hay, 1996; Lötscher and Hay, 1997). However, these sources of variation were not found at low levels of P

(Caradus *et al.*, 1998), suggesting that low P tolerance may not exist in the primary gene pool of white clover.

D. Tolerance to Biotic Stresses

Biotic stresses are a substantial constraint on the establishment, yield and longevity of forage legumes. White clover is usually perennial via a stoloniferous network, with an annual pattern of rapid growth in new stolons over spring and summer followed by deterioration in the winter. This is in contrast to the crown dominant forms of alfalfa and red clover, which rarely form rhizomatous roots. Across these plant perennation strategies, disease and pest loads are common in ecotype, landrace and some breeding material. In the case of virus infection, this may be conferred in natural populations to maintain genetic diversity through a genotype \times virus response interaction (Van Mølken and Stuefer, 2011) in white clover, where host plants have been shown to have differential response to virus loading.

Breeding efforts have focused alternately on tolerance to one or more biotic stresses, or resistance to particular pests and pathogens. Tolerance is an attractive breeding strategy, but may break down and additional stresses and their interactions weaken the plant. Resistance is also an attractive strategy, but is often not complete, simply inherited, or durable.

Operationally, the level of tolerance of forage synthetic varieties is measured by the proportion of tolerant plants. It differs, therefore, from the assessment based on the intensity of resistance that features genetically homogeneous varieties.

1. Alfalfa

Pest and disease pressure is a significant limitation on alfalfa production in many areas. The main diseases of alfalfa include four root diseases whose importance depends on regional areas: bacterial wilt caused by *Clavibacter michiganense* subsp. *insidiosum*; fusarium wilt caused by *Fusarium oxysporum*; verticillium wilt caused by *Verticillium albo-atrum*; phytophthora root rot caused by *Phytophthora medicaginis*; and the foliar disease anthracnose, caused by *Colletotrichum trifolii*. The primary evidence for genetic improvement through historic alfalfa breeding efforts has been shown to be in improving resistance or tolerance of specific biotic challenges contributing to persistence and yield stability, rather than yield potential *per se* (Lamb *et al.*, 2006). There are few interactions between forage quality and biotic stress tolerance and resistances in alfalfa (Fonseca *et al.*, 1999), indicating that selection for resistance or tolerance is not expected to negatively impact quality.

For Fusarium wilt, there has been some progress in developing field resistant types (Bozzini *et al.*, 2008). Verticillium wilt resistance is highly heritable, but remains hard to fix due to the autotetraploid genetics of alfalfa (Vandemark *et al.*, 2006). Phytophthora is a major problem in some regions of Australia. Research to dissect the genetic control of resistance in alfalfa has detected a number of QTL contributing to the resistance in a partial linkage map (Musial *et al.*, 2005). Response to se-

lection over a single cycle has been shown for host resistance to the winter fungal disease brown root rot, caused by *Phoma sclerotoides* (Hollingsworth *et al.*, 2005).

Efforts to map resistance to the foliar disease anthracnose have identified a multi-locus region of group 4 harbouring quantitative resistance factors contributing to a substantial resistance effect (Irwin *et al.*, 2006). Diseases often occur in complexes, making multiple resistances an important agronomic improvement objective. However, research has shown that in some cases only a low frequency of plants in a population harbor multiple resistance to both anthracnose and root rot (Mackie and Irwin, 1998).

The cultivation of widely heterogeneous varieties has provided substantial population buffering against genetic shifts in pathogen and pest populations. However, the current trend towards selection of more narrowly-based varieties could lead to greater chance of new pathotypes evolving (Irwin and Armour, 2014). This can be counteracted by deploying a diversity of resistances in new varieties through the development of molecular markers closely linked to resistance genes, as already pursued for anthracnose and phytophthora root rot by alfalfa breeding for sub-tropical Queensland (Irwin *et al.*, 2001).

Beyond these primary diseases, there has been substantial research on sclerotinia resistance with low to moderate heritability in pot systems (Kanbe *et al.*, 2002). Sources of resistance seem to have some adaptability, with shared resistances to *Sclerotium rolfsii* and *Sclerotinia trifoliorum* (Pratt and Rowe, 2002).

Beyond the fungal diseases, nematode resistance has been identified in both *sativa* and *falcata* germplasm (Julier *et al.*, 1996). Non-host resistance has been described for two root knot nematode species (*Meloidogyne chitwoodi* and *M. hapla*), where plant genetic characterization has shown them to be related mechanisms but not linked in terms of genetic loci underpinning the traits (McCord, 2012).

Pests are also a substantial constraint on forage or seed production. For some insects that are detrimental to forage production, tests have been developed to carry out breeding programs. These include the silverleaf whitefly (*Bemisia argentifolii*), for which partial resistance has been identified, acting on the first instar nymph stage (Jiang *et al.*, 2003). Tests for resistance to pea aphid (*Acyrtosiphon pisum*) (Berberet *et al.*, 1991; Girusse and Bournoville, 1994; Girusse *et al.*, 1998) and blue aphid (*A. kondoi*) have been established. Genetic variation among varieties was shown (Landré *et al.*, 1999), and the inheritance of resistance, mainly additive, was studied (Julier *et al.*, 2004). Resistance to potato leafhopper (*Empoasca fabae*) is related to glandular-haired genotypes (Elden and McCaslin, 1997). The mechanism of resistance to the potato leafhopper is probably not related to a physical constraint caused by the trichomes but to volatile compounds that decrease insect attraction for the resistant genotypes (Ranger *et al.*, 2005). Breeding for alfalfa weevil (*Hypera postica*) resistance has been efficient under field infestation (Busbice *et al.*, 1978), but a seedling test was further established (Ratcliffe and Elgin, 1987).

2. White clover

Breeding for plant health in white clover has largely focused on virus and nematode resistance. An isoflavone synthase has also been identified in white clover that may be manipulated to enhance isoflavonoid levels and provide insect resistance (Franzmayr *et al.*, 2012). Substantial improvement in white clover performance demonstrated when nematode burdens are removed from the soil (Mercer and Watson, 2007). The search for resistance has included evaluation of the secondary and tertiary gene pool (Hussain *et al.*, 1997; Mercer and Miller, 1997; Kouamé *et al.*, 1998) which identified multiple sources of resistance, including a single gene complete resistance in *T. semipilosum* (Barrett *et al.*, 2005a). Variation for resistance within white clover for root-knot nematode (*Meloidogyne* spp.) and clover cyst nematode (*Heterodera trifolii*) has also been found (Van Den Bosch and Mercer, 1996; Van Den Bosch *et al.*, 1997). Recurrent selection has proven successful in increasing those levels of resistance (Mercer *et al.*, 2000), and in lifting performance of selected lines in the field (Mercer *et al.*, 2005; Mercer *et al.*, 2008). Genetic analysis has revealed a combination of general combining ability (GCA) and specific combining ability (SCA) effects in the quantitative resistance in white clover to the southern root-knot nematode (Acharya *et al.*, 2011). Analysis of the root-knot resistance has shown it to be temperature sensitive (Mercer, 2005), breaking down at temperatures above 25°C.

Clover root weevil (*Sitona lepidus*) has been of particular concern in New Zealand for white clover and red clover, with a widespread search for tolerance or resistance revealing some potential for plant breeding options within an extensive survey (Crush *et al.*, 2005). While some virus may confer advantages on specific clover genotypes (Van Mólken and Stuefer, 2011), there is ample evidence of agronomic penalty (Jones, 2013). Genetic analysis has shown there is useful variation for virus resistance in white clover (Pederson and McLaughlin, 1994) which can be exploited through conventional means. However, a sustained effort to develop field tests (Smith *et al.*, 2007) and release white clover with engineered resistance to virus (Panter *et al.*, 2012; Forster *et al.*, 2013) has proceeded.

3. Red clover

Fusarium oxysporum is a major pathogen of red clover. Breeding efforts have shown moderate response to selection, with resistance characterized as quantitative with low heritability (Venuto *et al.*, 1999). Surveys of core collections have identified resistance (Vymyslicky *et al.*, 2012). *Sclerotinia trifoliorum* has only incomplete resistance, and response to selection was only realized for one cycle (Vleugels and van Bockstaele, 2013). Linkage mapping and QTL discovery has identified genome regions in linkage groups 3 and 6 contributing to resistance to *Fusarium* and *Sclerotinia* (Klimenko *et al.*, 2010).

Resistance to nematodes is another breeding objective, with root-knot (*Meloidogyne* spp.) and root-lesion (*Pratylenchus penetrans*) species both causing primary and secondary

pathologies. Efforts to characterize plant host resistance have identified quantitative additive genetic variation (Call *et al.*, 1997; Papadopoulos *et al.*, 2002). However further characterization and use of this variation for nematode resistance remains an understudied area. Resistance to bean yellow mosaic virus has been identified in core collection germplasm (Vymyslicky *et al.*, 2012).

Red clover formononetin levels have been shown to be a feeding deterrent against clover root weevil (*S. lepidus*) (Gerard *et al.*, 2005), however can have negative effects on ewe fertility thereby limiting the value of this as a selectable trait for resistance.

E. Tolerance to Grazing

Forage legumes can be grown in crop management systems that include both mechanical defoliation (mowing) and grazing, or either of them. Grazing can be rotational or continuous, either by sheep or cattle. The choice of legume species is largely dependent upon management system, expected duration of the pasture and the environment in which it is being grown. Grazing is widespread in extensive crop-livestock systems, where it minimizes feeding costs. As mentioned in Section I, tolerance to grazing is particularly important for sward persistence in these systems, especially when grazing is continuous, with high stocking rates and/or performed by sheep (Frame, 2005). Improved tolerance of grazing is therefore a breeding target for white clover, red clover and alfalfa, and is closely associated with the crop management system under which these different species are likely to be grown. Because of its stoloniferous growth habit, white clover is less affected by close, intense grazing than alfalfa and red clover (whose regrowth requires regeneration of new buds from the crown and recovery of root reserves). However, all three species display remarkable within-species variation for adaptation to severe grazing that is associated with specific morphological traits and tends to be inversely related to high yielding ability under a mowing regime (Evans *et al.*, 1992; Taylor, 2008; Annicchiarico *et al.*, 2010). In white clover, which is typically sown with a companion forage grass species, tolerance of grazing is closely associated with leaf size and associated stolon morphology (Abberton and Marshall, 2005). Small leaf types have a dense mat of small stolons that makes them particularly tolerant of intensive sheep grazing, whilst large leaf types have a less dense mat of larger stolons which can be easily removed by the grazing animal and are therefore more suited to conservation and cattle grazing systems (Frame, 2005). Between these two extremes are the medium leaf types that are more suited to rotational grazing by sheep and cattle. Productivity is greater with larger leaf size types but there is a general negative correlation between leaf size and persistency (Evans *et al.*, 1992)

Results in Table 4 for white clover material encompassing different leaf size classes indicate no genetic correlation for population yield responses across mowing and continuous sheep grazing regimes. This result supports the historical development

of specific varieties for these contrasting uses (Rhodes *et al.*, 1994), and suggests limited yield gains arising from indirect selection under a mowing regime targeted to intense grazing by sheep. However, breeding widely-adapted varieties has also been pursued with some success (Woodfield and Caradus, 1994), aiming to break the negative correlation between leaf size and persistence.

An alternative strategy for white clover breeding is introducing traits from closely related species that will improve tolerance to grazing. As considered in Section III, Caucasian clover is a very persistent and grazing-tolerant species (Taylor, 2008), as it grows and spreads within the sward by means of underground stems (rhizomes). The development of interspecific hybrids between white clover and Caucasian clover has been reported by a number of authors (Marshall *et al.*, 2004; Williams and Nichols, 2011). Abberton and Marshall (2005) summarized the introgression of the rhizomatous trait from Caucasian clover into white clover to increase the grazing tolerance of large leaf types. Thus far this has not resulted in commercially available varieties, but the first varieties from this program are in the last stage of official trials.

Alfalfa and red clover have different growth systems from white clover in that regrowth following defoliation is dependent upon the growth of new buds from the crown. In alfalfa, breeding for improved yield and persistence under grazing has shown substantial gains (Smith Jr *et al.*, 2000; Bouton and Gates, 2003) and is considered important to enable an expanded use of the crop. High-yielding, grazing tolerant alfalfa cultivars (e.g., 'Alfagraz') have been developed by selecting grazing tolerant plants from highly productive, multiple-pest resistant germplasm, selecting survivors from stands which have thinned appreciably after being grazed intensively by beef cattle (rather

than selecting based on an inferred grazing tolerant ideotype) (Bouton *et al.*, 1991; Bouton and Gates, 2003; Bouton, 2012). This selection procedure has also been used to develop grazing tolerant red clover cultivars. Cultivars developed from plants surviving continuous grazing are also more persistent under rotational stocking (Bouton and Gates, 2003; Brummer, 2006). Characteristics of grazing tolerant alfalfa appear to include the ability to maintain root energy reserves under frequent defoliation, the presence of leaves low in the canopy below the grazing height, and an increased number of crown buds (Brummer and Bouton, 1991; Brummer and Bouton, 1992).

Most grazing-tolerant alfalfa cultivars belong to the deep-crowned, semi-erect plant type indicated as 'D4' in Figure 4 (Pecetti *et al.*, 2006), whose tolerance tends to rely on conspicuous shoot proliferation from a broad crown. They are well-suited to cattle rotational grazing. Creeping-rooted varieties ('D2' type in Figure 4) have also been bred but with limited commercial success, mainly because of the erratic expression of the creeping trait (Pecetti *et al.*, 2004). The prostrate 'D1' type in Figure 4, rhizomatous with great sideways-spreading ability, is promising for further tolerance improvements. Recent 'D1' selections have shown lower yielding ability under mowing but better adaptation to continuous sheep grazing than 'D4' types, being also valuable under cattle grazing (Annicchiarico *et al.*, 2010). Challenges for improving 'D1' material are represented by high winter dormancy and lower seed yield than 'D4' types, both consequences of their *falcata* background (Pecetti *et al.*, 2008). Their hybridization with semi-erect or erect alfalfa material could produce semi-prostrate material ('D3' type in Figure 4) with broad adaptation to grazing systems and climatic conditions, along with good seed yield (ideally combining these features more effectively via marker-assisted selection). An alternative

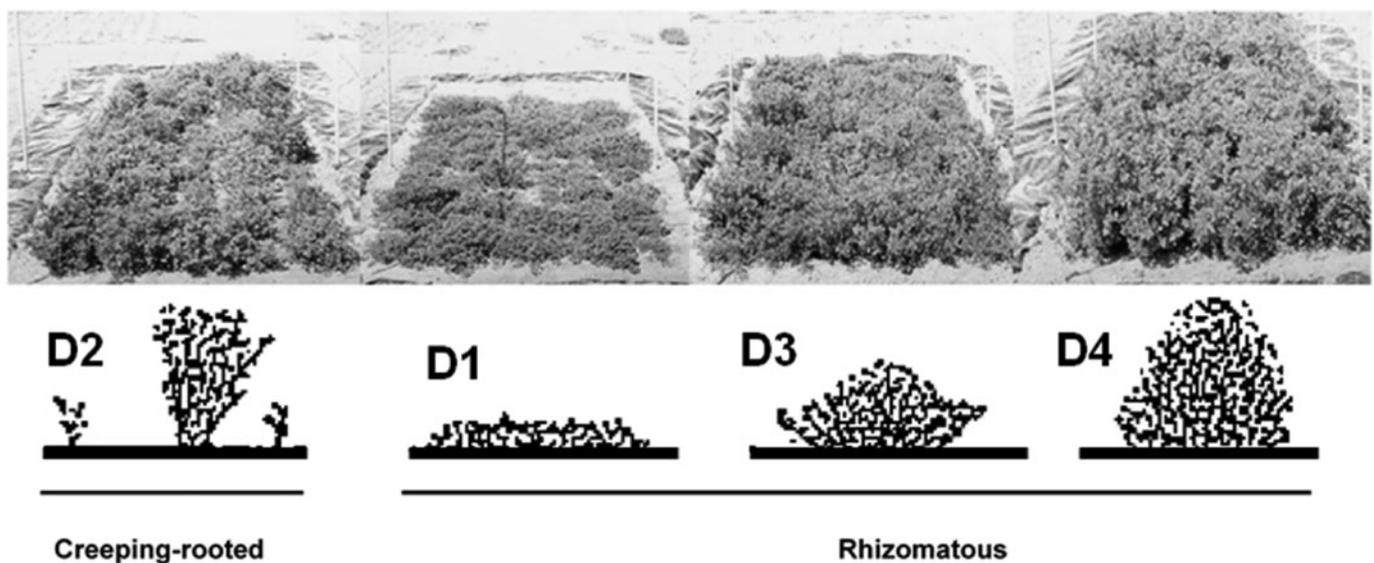


FIG. 4. Deep-crowned morphophysiological plant types within *M. sativa* subsp. *sativa*, *falcata* and *varia* (modified from Piano *et al.*, 1996).

strategy for further improvement of alfalfa grazing tolerance is the recurrent selection of 'D4' types under severe, continuous grazing.

Red clover generally lacks persistence when grazed (Frame, 2005) and consequently improved persistence is a major breeding target for this species (Riday, 2009), with performance under grazing a key element (Ford and Barrett, 2011; Marshall *et al.*, 2012). Efforts to improve grazing tolerance have focused on the effect of variation in plant morphology including crown structure (Ortega and Rhodes, 1996), and selection for crown survival. This latter approach has been shown to improve plant persistence, leading to higher red clover dry matter yields in the third harvest year (Marshall *et al.*, 2012). Taylor (2008), in his review on clover breeding developments in the United States, highlighted that some red clover types possess the ability to develop adventitious roots (Montpetit and Coulman, 1991) and also stolons (Smith and Bishop, 1993), both of which have the potential to improve grazing tolerance. In a recent comparison of novel germplasm with cultivars currently available in New Zealand, genetic material with improved persistence under cattle grazing was identified (Ford and Barrett, 2011) but further experiments are required to ascertain the basis of the improved persistence and quantify persistence under different management systems including sheep grazing.

F. Forage Quality

1. Quality and crop utilization

Forage legumes such as white clover, red clover and alfalfa have a high value for ruminant production, owing to their high protein content (usually between 15 and 20% on a dry matter basis for alfalfa and red clover, and up to 30% for white clover), high buffering effect (which reduces the risk of acidosis), and fairly high energy content (Frame, 2005). Their high protein content relative to the energy content can lead to high protein degradation in the rumen. However, when they are combined with energy-rich forage (such as perennial grasses or corn silage) they provide a well-balanced protein/energy diet that is suitable for highly-productive animals, with little requirements of energy or protein supplementation.

They can be fed to animals through grazing or after being mown (as hay, silage, or fresh forage). For grazing, rapid protein degradation can lead to the built-up of stable foams and cause life threatening bloat, particularly in cattle. In mixed swards with ryegrass, the forage quality of white and red clover and alfalfa depends upon the legume species in the mixture and the management system under which they are being grown (Gierus *et al.*, 2012). Nevertheless there is good evidence that the forage quality of mixed grass/legume swards can provide significant production benefits and limit bloat risk when grazed. It has been shown that red clover for silage and white clover in grazed swards can lead to enhanced growth rate and milk yield in comparison with pure grass swards primarily due to enhanced

intake, as digestibility is comparable with grasses (Dewhurst *et al.*, 2009).

The potential for genetic improvement of forage quality traits has recently become an increasingly important target for forage breeding programs. The focus on forage quality improvement has coincided with recent improvements in animal production potential through the selection of high genetic merit herds and flocks; as animal genetics develop, so must the genetics of forage crops in order to match animal requirements. Forage crop breeding targets are also being informed by increasing knowledge about the effect of forage quality on animal performance and the impact of diet on rumen function and the environment, particularly in relation to balancing nitrogen and energy in the rumen of ruminant animals (Kingston-Smith *et al.*, 2013). Understanding the mechanisms and impact of such rumen processes has been aided by selective forage breeding, which has produced a wide range of germplasm with different forage quality characteristics and agronomic attributes (Abberton *et al.*, 2008) for use in animal production studies. These studies have shown that increased diet efficiency could be achieved by altering the rumen degradability of forage protein to reduce its availability to rumen microbes, thereby better matching the availability of soluble carbohydrates (energy) present (Kingston-Smith *et al.*, 2013). Strategies include increasing the amount of readily available energy accessible to match available nitrogen sources or to provide a level of protection to the forage proteins, reducing the rate at which their breakdown products are made available to the colonizing microbial population. Unfortunately, protein degradability of alfalfa, white clover and red clover is generally high. The forage quality characteristics of these species and some of the breeding approaches being used to improve forage quality are summarized in the following subsections.

2. Alfalfa

Alfalfa is considered one of the best forage crops for feeding ruminant animals because of its high nutritional value (Sanders *et al.*, 2011; Yari *et al.*, 2012). Its leaves are protein- and vitamin-rich and low in cell wall concentration and therefore highly digestible. In contrast, stems exhibit low digestibility as a result of high concentrations of cell wall polysaccharides and lignin (Schnurr *et al.*, 2007). Forage quality (as expressed by higher values of digestibility and protein content, and lower values of neutral- [NDF] and acid-detergent fibre [ADF] and acid-detergent lignin [ADL]) is strictly associated positively with leaf/stem ratio and negatively with the degree of stem lignification, both becoming less favorable with increased plant maturity and progression of the reproductive stage. Thus, maturity stage and mowing time have a significant effect on the main forage quality parameters (Yari *et al.*, 2012). Non-structural carbohydrate content is low but increases throughout the day, so a harvest at the end of the day has a high energy content and a slight decline in protein content (Morin *et al.*, 2011). Increase in energy content had a positive impact of N utilization by lactating cows (Brito *et al.*, 2009).

Breeding for improved quality can exploit the wide among-population and within-population variation existing for leaf/stem ratio and major quality parameters (Julier *et al.*, 1996; Julier *et al.*, 2000; Annicchiarico, 2007a). On the other hand, narrow-sense heritability values for these traits are reportedly modest, ranging from 0.18 to 0.39 (Guines *et al.*, 2002). Selection for improved quality has produced varieties with consistently greater digestibility and protein content and lower NDF (Hall *et al.*, 2000). The development of increasingly reliable near-infrared reflectance spectroscopy (NIRS)-based estimation has made possible and cost-effective the evaluation of many plants or families in breeding programs.

Strategies to improve forage quality of alfalfa include: (i) the selection for individual quality parameters; (ii) morphological changes aimed to increase the leaf/stem ratio, such as the multifoliate trait (which suffers from inconsistent expression: Juan *et al.*, 1993), and the selection for increased node number and decreased internode length (Annicchiarico *et al.*, 2010); (iii) selecting for adaptation to early mowing (e.g., at 5% blooming), to avoid harvesting late-stage, lignified stems (Rotili *et al.*, 1994); (iv) the selection of transgenic varieties with modified lignin composition (Getachew *et al.*, 2011), obtained by down-regulated biosynthesis of the S lignin unit (Guo *et al.*, 2001b).

Selecting for later reproductive development can provide material with an extended useful period for harvesting high-quality forage and/or suitable for reducing the total number of harvests per year. This latter feature is highly valued by farmers, when considering that the main foreseen use of forthcoming transgenic varieties in the United States is for 8–12 days delayed harvesting of forage with the same digestibility as that of earlier-harvested conventional varieties, rather than harvesting more digestible forage at conventional, earlier harvest dates (Undersander *et al.*, 2009).

3. White clover

White clover has outstanding digestibility and protein content, and relatively low levels of water soluble carbohydrate (WSC) (Beever, 1993; Marshall *et al.*, 2004). However, studies suggest that the high protein content of white clover may contribute to the inefficient utilization of nitrogen in mixed forage in the rumen that leads to pollution problems, such as the release of greenhouse gases and ammonia volatilization (Waghorn and Caradus, 1994). Widdup *et al.* (2010) demonstrated a rapid response to selection for increased WSC, accompanied by decreased protein concentration. That response was shown to be stable across seasons and locations in a range of breeding populations. A current target of the IBERS white clover breeding program is to quantify the extent of white clover genetic variation for protein content with a view to developing varieties with a protein content that better matches the energy supply of the companion grass. In related research, Marshall *et al.* (2004) quantified the forage quality characteristics of interspecific hybrids between white clover and *T. ambiguum* and showed that the backcross hybrids had a higher WSC content, lower N and

comparable digestibility. The authors suggested that this represented potential improvements of the behavior of white clover as a legume grown with grass for both grazing and silage.

A key consideration in the utilization of clovers for grazing is the bloat risk. There is increasing awareness of the importance of condensed tannins (CTs) or proanthocyanidins that stabilize proteins in the rumen and prevent bloat, with associated environmental benefits through reduced losses of N. CTs are absent from the leaves of white or red clover but present in their inflorescences. Exploiting the CTs in inflorescences is one approach that has been studied (Burggraaf *et al.*, 2008). Inclusion in mixtures of a species such as birdsfoot trefoil (Julier *et al.*, 2003; Collins *et al.*, 2006; Abberton *et al.*, 2008), which contains high levels of CTs in its leaves, is an alternative strategy which requires the development of varieties of this species which can persist in mixed swards (Marshall *et al.*, 2013). Transgenic approaches to modify white clover to produce proanthocyanidins and CTs have also been considered (Forster *et al.*, 2013). They have been markedly advanced by the transcription factor discovered and cloned by Hancock *et al.* (2012), proven to enable expression and accumulation of CTs in alfalfa and white clover.

Cyanogenic potential is a trait of adaptive significance in white clover wild populations (Williams, 1987a). Its desirable level in white clover varieties is somewhat controversial (Lehmann *et al.*, 1991; Crush and Caradus, 1995), and depends to some extent on the target environment and utilization. Highly cyanogenic material may affect grazing animals negatively through cyanide poisoning or indirect effects of cyanide and thiocyanate on iodine, selenium and sulphur metabolisms (Gutzwiller, 1993). However, cyanogenic germplasm tends to display greater persistence than acyanogenic material in many environments, because of greater plant resistance (non-preference) to various species of leaf feeding insects and mollusks (Raffaelli and Mordue, 1990). Breeding acyanogenic varieties can be based on landraces and wild populations of Ladino and smaller leaf types originating in northern Italy (Annicchiarico, 2012), as well as on ecotypes from the Netherlands and some north-European countries (Caradus *et al.*, 1989).

4. Red clover

Red clover has high protein content but contains little soluble carbohydrates and tends to be less digestible than forage grasses. This is easily managed by growing red clover with highly digestible grasses rather than in a monoculture (Boller *et al.*, 2010b). Genetic variation for degradability of protein in the rumen has been reported (Broderick *et al.*, 2004). Current research to improve forage quality is largely focused on secondary plant metabolites. Red clover contains high levels of oestrogenic compounds that when fed to ewes before mating can reduce conception. Breeding red clover for a low level of formononetin is the objective of breeding programs in Europe and New Zealand (Rumball *et al.*, 2005). More recently there has been considerable interest in the genetic variability in the PPO enzyme (polyphenol oxidase) which is present and active

in red clover leaves and how it impacts on N utilization. There is good evidence that compared to alfalfa and white clover, red clover seems to positively affect N utilization, which may be related to the activity of PPO (Eickler *et al.*, 2011). The PPO mechanism may effectively reduce protein breakdown in silage (Sullivan and Hatfield, 2006) and result in over 80% of the protein in red clover silage being retained as 'true protein,' providing an improved nitrogen use efficiency and product quality when fed to cattle (Winters and Minchin, 2002). The improved N use efficiency (higher milk N/N intake) and lower urinary N excretion of dairy cows when fed red clover silage in comparison with alfalfa silage has been attributed to PPO activity (Broderick *et al.*, 2007). The inhibitory effect of PPO activity on proteolysis and also lipolysis (Lee *et al.*, 2006) adds support for a strategy to breed improved red clover varieties with increased PPO activity (Kingston-Smith *et al.*, 2013). Such work is ongoing but provides interesting opportunities for the future.

G. Seed Yield

1. Alfalfa

Seed yield is a vital trait for the commercial success of a forage cultivar, and is of ongoing value in systems where re-seeding contributes to the renewal of pasture through seed fall or ingested seed via grazing or hay, and redistribution in dung. Interestingly, despite the commercial value to the seed industry, landraces have been shown to have similar or superior levels of seed yield as compared to bred cultivars (Annicchiarico *et al.*, 2007). Pollination interactions are complex, and recent evidence highlights the relatively high levels of self-pollination and non-uniform distribution of male fertility and pollen flow in alfalfa breeding systems (Riday *et al.*, 2013).

There is extensive variation for seed yield among and within cultivars (Bolaños-Aguilar *et al.*, 2000). Seed yield per plant exhibited narrow-sense heritability close to 0.50, and could effectively be selected for by selecting for seed yield per inflorescence that can be scored in the planting year (Bolaños-Aguilar *et al.*, 2001). Raceme length has also been identified as a simple correlated trait to select for seed yield, as it is correlated with seed per raceme and pod number (Bodzon, 2004). The identification of ovule sterility close to 35% and its range of variation (Wang *et al.*, 2011) suggest there may be substantial opportunity to improve seed yield by addressing this trait, which is quantitatively inherited (Rosellini *et al.*, 1998).

Seed yield can display large cultivar \times environment interaction (Bolaños-Aguilar *et al.*, 2002) which parallels that for forage yield responses (Annicchiarico *et al.*, 2012), as well as only moderate genetic correlation for genotype responses across spaced plant and dense sward assessments (Annicchiarico, 2006b). Seed yield was found to be highly correlated positively to above-ground biomass at seed harvest stage (Bolaños-Aguilar *et al.*, 2002), indicating that genetic improvement of seed yield and forage yield are not antagonistic in this species.

2. White clover

White clover seed yield is not high, however pollen availability and the self-incompatibility system have been ruled out as causes of low seed yield (Cowan *et al.*, 2000). Selection for seed yield is favoured by extensive genetic variation in adapted germplasm (Jahufer and Gawler, 2000) and moderately high narrow-sense heritability for the trait (0.60) and its most-related components, i.e., inflorescence number, florets per inflorescence and seeds per floret (in the range 0.58–0.70) (Annicchiarico *et al.*, 1999). However, selection for seed yield is hindered by its inverse genetic correlation with both forage yield potential and general persistence as provided by stolon production (Annicchiarico *et al.*, 1999). Based on this information, phenotypic selection has been shown to be effective in improving seed yield while maintaining competitive agronomic performance (Widdup *et al.*, 2004; Woodfield *et al.*, 2004), overcoming the economic threshold for forage seed yield in white clover in New Zealand.

High production of inflorescences has frequently been reported as the main seed yield component trait (Thomas, 1987; Jahufer and Gawler, 2000). However, its physiologically-based negative relationship with production of stolons (i.e., the main traits associated with general persistence) suggests to improve seed yield by increasing other seed yield components. The product of inflorescence number and florets per inflorescence can provide a low-cost, effective indirect selection criterion for seed yield (Annicchiarico, 1997). Selection for yield per inflorescence has been shown to be effective and does not change stolon characteristics (Woodfield *et al.*, 2004). Selection for peduncles that are thick, strong and distinctly taller relative to the vegetative canopy contributes to higher seed yields by minimizing seed losses at mechanical harvest and facilitating pollination (Marshall, 1995). Interspecific crosses between white clover and *T. nigrescens* aimed at introducing the profuse flowering ability of *T. nigrescens* (Marshall *et al.*, 2008).

QTL analysis identified two genome regions harbouring major QTL (Barrett *et al.*, 2005b). Subsequent analysis revealed substantial effects in a number of breeding material surveyed with markers sourced from those genome regions, with 8 out of the 12 populations surveyed showing marker effects accounting for a mean 38% differential in seed yield (Barrett *et al.*, 2009), leading to targeted application of the marker assay in selecting for seed yield in white clover breeding populations.

3. Red clover

Relatively less research is reported in red clover seed yield, despite it being a primary limitation on red clover seed production and variety commercial success in some regions, particularly for tetraploid varieties. Low seed yield is due in part to poor ovule fertility (Buyukkartal, 2008). Lower seed yield in tetraploid varieties than in diploid ones seems to be related to lower pollination. Indeed, the corolla is longer so that only long-tongued bees efficiently visit the flowers (Tasei, 1984). Pollinator interactions can be important in red clover (Wermuth

and Dupont, 2010). QTL analysis has identified a number of genome regions (Herrmann *et al.*, 2006) contributing to genetic control of the trait, which exhibits moderate to high heritability.

H. Other Traits

Non-feed uses of forage legumes include biomass energy production, phytoremediation, and production of positive impacts on the environment.

Biomass production for energy purpose could be more efficiently obtained with alfalfa than with clovers (although red clover might have an interest in some high latitude regions). Energy production requires high cell wall polysaccharides but low nitrogen content. A dual-purpose alfalfa crop has been proposed based on the separate harvest of leaves and stems, using the leaves to produce high-protein feedstuff and the stems to produce electricity, biofuel, or bio-oil. The specific crop management for this utilization implies longer regrowth periods to increase both stem and leaf yields, and lower plant density to reduce leaf senescence (Lamb *et al.*, 2003). Germplasm specifically adapted to this use has been selected for erect growth habit and thick, non-lodging stems (Lamb *et al.*, 2007).

Phytoremediation uses plants to remove, degrade or immobilize the soil contaminants through physiological processes. Deep rooted species that produce high biomass yield are most efficient in capturing the contaminants. Nitrogen pollution in ground water is very common because of over-fertilized crops. Alfalfa has been shown to catch nitrate deeply in the soil (Blumenthal and Russelle, 1996) and to efficiently remove excess inorganic nitrogen from the soils (Russelle *et al.*, 2001). The application of manure slurry to an alfalfa crop induces limited nitrate leaching (Thiébeau *et al.*, 2004). An N₂-fixing alfalfa variety removed nearly 60% more soil and manure N than an ineffective nodulated population (Russelle *et al.*, 2007). A method to create varieties with improved soil nitrogen uptake has been established (Lamb *et al.*, 2008)

The role of forage legumes for *in situ*-remediation of soils polluted by phthalate esters (Ma *et al.*, 2013), crude-oil (Zhong *et al.*, 2012) or dioxin (Wang and Oyaizu, 2011) has also been described. The enhancement of microbial activity in the rhizosphere of legume species seemed to be a positive aspect contributing to remediation. No trait that could be specifically improved has been described yet.

As reported in Section I, perennial forage legumes produce beneficial environmental effects, including the production of large amounts of nitrogen through N fixation (up to 400 kg N/ha/year; Angus and Peoples, 2012) that is partly allocated to the rooting system (110 to 165 kg N/ha; Justes *et al.*, 2001). This nitrogen enters a mineralization phase and is progressively available to the following crop. The deep rooting system of alfalfa benefits the following crop by improving soil structure, water infiltration, nitrogen diffusion, microbiological activity, and nutrient cycling. In addition, alfalfa and other forage legumes contribute to biodiversity of insects, small mammals and birds by providing a key habitat, especially when adopting environment-

friendly agricultural practices (Berthet *et al.*, 2012). No trait of specific interest for this use has been reported yet.

V. GENOMIC RESOURCES

The development of genomic resources for forage legume species has lagged behind that of other species, because of their genome complexity, heterozygosity and polyploidy. Other legume species grown for grain, such as pea (*Pisum sativum*) and faba bean (*Vicia faba*), are diploid, often with a homozygous genome. However, most grain legume species also have larger genome size (e.g., 4.5 pg for pea, and 13.1 pg for faba bean) than alfalfa (1.7 pg - 1,692 Mbp for 1 C), white clover (0.5 pg - 518 Mbp) and red clover (1.1 pg - 1,093 Mbp) (Bennett and Leitch, 2011). *M. truncatula* was chosen in the 1990's as a model species to develop genomic resources and tools that would be available to all legume crops, because it is diploid, autogamous, with a small genome (0.5 pg - 579 Mbp for 1 C, for cv. Jemalong) and a short life cycle (Young *et al.*, 2005). This species belongs to the Galegoid clade within the Papilionidae subfamily, like alfalfa, clovers, pea and faba bean.

Forage legume crops are orphan species regarding the development of genomic resources. *M. truncatula* sequencing and genome assembly has given crucial resources (genome structure, gene sequences, markers) for legume crops. Next-generation sequencing technology offers the possibility to directly sequence the genome of forage legume species and to analyze their genomic polymorphism. Although challenging, this objective will definitely contribute, along with the development of high-throughput phenotyping technologies, to introduce genomics into the breeding schemes of commercially successful novel varieties.

A. Genomic Resources in *M. truncatula* and Synteny with Forage Legume Species

Genetic resources, mutants, mapping populations, markers and EST sequences have been developed in the past 20 years. The gene-rich portions of the *M. truncatula* genome (genotype A17) have been sequenced using the Sanger technology on BAC libraries, in combination with physical and genetic mapping. A high quality genome sequence assembly was obtained. It was anchored to chromosomes and covered about two thirds of *M. truncatula* genes (Young *et al.*, 2011). In addition to this data, next-generation sequencing methods were used to complete genome sequence. The new genome assembly covers nearly 95% of *M. truncatula* genes, and version 4.0 of the *M. truncatula* sequence is now available (<http://medicago.jcvi.org/medicago>). Gene annotation is also available, with 31 661 high confidence gene loci and about the same number of putative genes (Young *et al.*, 2011). The comparison of this analysis of *M. truncatula* with those for other legumes species that have been sequenced [*Lotus japonicus*, *Glycine max* (soybean), *Cajanus cajan* (pigeon pea), *Cicer arietinum* (chickpea) and *Phaseolus vulgaris* (common bean)] showed that an ancient whole genome duplication (around 58 million years) followed by genome rearrangements

and gene losses occurred before speciation within the Papilionidae subfamily (Young *et al.*, 2011).

The analysis of sequence diversity in *M. truncatula* is currently under study (<http://www.medicagohapmap.org/?genome>). A set of 384 genotypes covering the range of genetic resources are being sequenced using next-generation sequencing tools, in order to detect single nucleotide polymorphisms (SNP), insertions/deletions (INDEL) and copy number variants (Branca *et al.*, 2011). Various studies conducted in *M. truncatula* on linkage disequilibrium (Branca *et al.*, 2011) or analysis of recombination rate along the genome (Paape *et al.*, 2012) are providing better insight of genome structure and evolution which will help in further genetic analyses.

Genomic and genetic resources of *M. truncatula* can be useful to forage legume breeders. In the 2000's, the EST sequences were used to develop SSR markers on *M. truncatula* and about 50% of these markers showed amplification and polymorphism in a biparental cross of alfalfa (Julier *et al.*, 2003). They were used to build genetic maps in alfalfa (Julier *et al.*, 2003; Sledge *et al.*, 2005) and to analyse population structure and heterozygosity (Flajoulot *et al.*, 2005). A large proportion of these markers also showed amplification and polymorphism in clovers (Zhang *et al.*, 2007; T. Huguet, unpublished data), but marker development was also specifically carried out in these species (see below).

In many cases, the traits that are of agronomic interest in crop species can be observed in the model species and show genetic variation. Their genetic determinism may be studied by using various methods: QTL mapping, association genetics, mutants, transcriptomics, etc. The genomic resources help to identify the genes involved in trait variation and to study their expression and regulation. The genes that are identified in such studies become candidates to explain variation in crop legume traits (see section VI). There might be a definite limit, however, to the usefulness of information issued from *M. truncatula* for improving forage yield, persistence and tolerance to abiotic stresses (drought; low temperatures) of perennial legume species, when considering the wide differences between an annual and a perennial species for key adaptive traits (e.g., winter dormancy, or nitrogen concentration of root reserves for regrowth and sugar reserves for frost tolerance: Volenec *et al.*, 2002). In addition, metabolites produced by same genes may have different functions in *M. truncatula* and *M. sativa* (Volenec *et al.*, 2002), while differences due to ploidy level may further contribute to inconsistent genetic control of some morphophysiological traits between the two species (Bingham *et al.*, 1994).

Interest in model legume sequences from forage legume geneticists strongly relies on the synteny between model and crop species. Synteny is the preservation of gene order in two species and can be analysed at different levels: macro-synteny at the level of chromosomes and micro-synteny at the level of genomic regions. Evaluation of macrosynteny was carried out either with markers (Choi *et al.*, 2004b) or after genome sequencing (Young *et al.*, 2011). The ancestral duplication has

complicated the analysis, but a clear macrosynteny has been observed within the Papilionidae subfamily. Micro-synteny, defined as the maintenance of gene order in a genome region of about a BAC, was also high when comparing *M. truncatula* with *L. japonicus* and *G. max*. This suggests that both macro and micro-synteny are high among *M. truncatula* and forage legume species that belong to the Galegoid clade within the Papilionidae subfamily. Indeed, by using molecular markers originating from ESTs, a high colinearity of markers was found between *M. truncatula* and alfalfa (Julier *et al.*, 2003; Choi *et al.*, 2004a). Recent data on the genome sequence of alfalfa was assembled and compared to the *M. truncatula* genome (Julier *et al.*, 2014). Almost half of the *M. truncatula* genes were recovered in the alfalfa genome assembly. At the gene level, the sequences of the exons were highly conserved, but the sequences of the introns were not conserved among the two species. Further analysis on the alfalfa genome data set will complete the comparison of the two genomes.

In silico referencing of white clover to *M. truncatula* has identified macro-syntenic relationships maintained between these two species (George *et al.*, 2008; Isobe *et al.*, 2012), supported by evidence from mapped comparative markers (Zhang *et al.*, 2007). A recent study (Griffiths *et al.*, 2013) shows a possible inter-chromosomal rearrangement where chromosomes 2 and 6 of *M. truncatula* were split across chromosomes 2 and 6 of white clover. The micro-synteny, also studied *in silico* (Hand *et al.*, 2010), seemed high as well, even if some genome regions from *T. repens* were not recovered in the former 3.0 version of the *M. truncatula* genome that was used. In this allotetraploid species, the issue of macro-synteny between the two genomes is also important. The two homoeologous genomes 0 and P' showed no chromosomal rearrangements, and identity and similarity between homoeologous genes were high (Hand *et al.*, 2010; Griffiths *et al.*, 2013).

As in white clover, the order of the markers on a genetic map of red clover was compared to the *M. truncatula* genome with *in silico* alignment (Sato *et al.*, 2005). The *M. truncatula* genome had not been completed in 2005, preventing the observation of a segmental macro-synteny and a possible micro-synteny. The distribution of the genome between the eight linkage groups of *M. truncatula* and the seven of red clover has not yet been clarified. The direct comparative mapping of alfalfa, white clover and red clover (Zhang *et al.*, 2007) also showed high macro-synteny.

The *M. truncatula* genome sequence is thus a reference sequence for legume species. Next-generation sequencing now enables sequencing of forage legume species, but their genome assembly is challenging because of heterozygosity. The *M. truncatula* genome will probably simplify the genome assembly of crop species (Young and Udvardi, 2009).

B. Alfalfa

The development of genomic data in alfalfa has been slow for two reasons: its phylogenetic proximity with the model species *M. truncatula*, which provided substantial data directly usable

on alfalfa, and its tetrasomic inheritance, which was expected to complicate the interpretation of sequence data. No BAC library has been produced, but 12,561 ESTs are deposited on the NCBI website (as on 09/09/2013). Most of them come from two studies, an alfalfa aluminum suppression subtractive library (X. Li, unpublished), and a library of glandular trichome of alfalfa stems (G. Wang, unpublished). Recently, transcriptome sequencing of two alfalfa genotypes has been reported (Han *et al.*, 2011) in which more than 50,000 unique sequences were identified. Among them, 14,753 are deposited on the NCBI website. At INRA (France), transcriptome sequencing has also been carried out on 10 individuals, either cultivated or wild (N. Chantret, pers. comm.). Genome sequencing programs have started in France and the United States. In France, sequencing of one genotype is based on paired-end and mate-pair data obtained on Illumina Hi-Seq. Up to now, *de novo* assembly of these data was not very successful in this autotetraploid species, probably because of important allelic diversity (up to four different haplotypes), with large indels. Current sequence assembly thus relies on the *M. truncatula* sequence, considering alfalfa sequencing as a complex resequencing of the model species. Microassembly, based on mapping alfalfa reads on *M. truncatula* gene sequences, was thus attempted. With available alfalfa data, about 47% of *M. truncatula* genes were recovered (Julier *et al.*, 2014). However, if exon regions were highly conserved, non-coding regions were not conserved in the two species. In addition, some of the genes may not have been recovered because of large sequence polymorphism in the two species.

The first SSR markers to be developed on alfalfa derived from a genomic library, but only 10 markers were defined (Diwan *et al.*, 1997; Diwan *et al.*, 2000). Recently, the EST sequences available on the NCBI were tested for SSR motifs (Wang *et al.*, 2013), and 716 ESTs carried a SSR. One hundred primer pairs were defined and 29 markers showed a polymorphic pattern. In fact, most SSRs used on alfalfa were derived from *M. truncatula*. The SSRs initially developed on *M. truncatula* were simply tested for amplification and polymorphism in alfalfa, and most of them revealed amplification and polymorphism (Julier *et al.*, 2003; Sledge *et al.*, 2005). Between 80% (Julier *et al.*, 2003) and 64% (Sledge *et al.*, 2005) of the SSRs developed from *M. truncatula* ESTs showed amplification on two parents of an alfalfa mapping population. This rate of amplification was lower when the SSRs were developed from BAC sequences, reaching less than 50% (Sledge *et al.*, 2005). The high conservation of gene sequences between alfalfa and *M. truncatula*, especially for exons, explain why primers developed into *M. truncatula* ESTs gave amplification in alfalfa. Conversely, the low conservation of non-expressed sequences when comparing *M. truncatula* and alfalfa explains why the primers developed from BAC sequences may not amplify alfalfa fragments. The rate of polymorphism, i.e., the proportion of EST-SSR markers that were polymorphic among two parents of a mapping population, reached 50% (Julier *et al.*, 2003; Sledge *et al.*, 2005). The large number of ESTs available in *M. truncatula* and this high

rate of success when testing EST-SSRs from *M. truncatula* in alfalfa indicate the presence of a large reserve of SSR markers for alfalfa mapping or diversity studies.

The identification of SNPs is now possible. With a shotgun sequencing of cDNA of two alfalfa genotypes, 40,661 candidate SNPs distributed throughout the genome were found (Han *et al.*, 2011). Genotyping the SNPs requires an adequate method. HRM (High Resolution Melt) markers were developed by defining primers around the SNPs and showed clear and legible patterns (Han *et al.*, 2012). The parental genotypes and their progeny were identified from melting curve profiles. In another study based on transcriptome sequencing of 27 alfalfa genotypes, 0.9 million SNPs were discovered (Li *et al.*, 2014) and about 10,000 were used to develop an Illumina Infinium array. Genotyping of a large set of individuals was successful and some SNPs gave access to the five allelic dosage classes.

Genotyping by sequencing might be the ultimate solution for alfalfa genotyping. At the laboratory level, the question will be how to reduce the complexity of the genome. Two choices are possible: sequencing of genomic DNA submitted to an appropriate restriction enzyme, or sequencing of cDNA with an adequate combination of organs, stages and growing conditions in plant samples. One key point might be the bioinformatic programs that are needed to go from the raw sequence data to the genotyping data in this autotetraploid species.

C. White Clover

Genomic data have been generated in white clover for molecular marker discovery, gene discovery, partial genome assembly, and gene expression analysis. Sources include a methyl-filtered library (Griffiths *et al.*, 2013) and an earlier EST library (Sawbridge *et al.*, 2003). A recent next-generation sequence resource using inbred genotypes in addition to a panel of diverse clover plants has been developed and used for SNP discovery (Nagy *et al.*, 2013).

Marker development from targeted sequence can identify polymorphism based on sequence identity (e.g., SNP) or length, such as SSR arrays. In the absence of a full reference genome for white clover and progenitors, homoeologous sequence similarity in genic regions hinders development of an efficient SNP discovery process. This is predominantly due to a high proportion of putative SNP markers *in silico* arising from conflation of orthologous sequence within homoeologous pairs (Cogan *et al.*, 2007). Reference sequence from progenitor species (Ellison *et al.*, 2006) partially overcomes this limitation (Hand *et al.*, 2008). A recent report (Nagy *et al.*, 2013) demonstrates a novel approach using sequence data of diverse genotypes combined with sequence analysis and haplotyping of two inbreds as a pseudo-double haploid to identify over 70,000 putative SNPs in white clover, without reliance on a progenitor reference sequence (Nagy *et al.*, 2013).

Polymorphisms in candidate gene sequence can be used as markers with potential functional effects to enrich maps and advance the genetic dissection of some traits (Gupta and Rustgi,

2004). Although a relatively laborious process, sequencing individual candidate genes can be used to identify haplotypes and overcome the limitations of *in silico* SNP discovery experienced in sequence databases (Cogan *et al.*, 2007).

Markers using SSR polymorphism are a co-dominant system that is proven, transportable, amenable to semi-automated assay, moderately cost-effective, and scalable. SSR markers have been used in a number of applications in plant improvement (Kalia *et al.*, 2011), and are estimated to occur at a density of one per 4.7 kb in transcribed regions of the white clover genome (Isobe *et al.*, 2012). Some effort has been made to identify homoeologue sets based on sequence data from the putative progenitor species *T. occidentale* (Hand *et al.*, 2008; Wang *et al.*, 2010). Large mapped (Isobe *et al.*, 2012) and unmapped (Zhang *et al.*, 2008) sets of white clover SSR markers have been made publicly available to augment three small sets of mapped SSR markers (Kölliker *et al.*, 2001a; Barrett *et al.*, 2004; Febrer *et al.*, 2007). In addition, substantial marker and linkage map resources in red clover (Isobe *et al.*, 2009) have been applied in white clover and *T. subterraneum* for comparative mapping (Zhang *et al.*, 2007; Ghamkhar *et al.*, 2012; Isobe *et al.*, 2012).

A comprehensive, integrated genetic linkage map (Griffiths *et al.*, 2013) is now available, based on SSRs and candidate genes. This integrated genetic linkage analysis and its component maps provide a consistent and comprehensive view of the white clover genome, with alignment to *M. truncatula*. Associated marker locus information, particularly the homoeologue-identifying SSRs, offers a new resource for forage legume research enabling genetic analysis and improvement of this prominent species. This integrated map is predated by four published independent genetic linkage maps of varying completeness and quality based on *Trifolium* SSR (Jones *et al.*, 2003; Barrett *et al.*, 2004; Zhang *et al.*, 2007; Isobe *et al.*, 2012).

The intersection of genotyping and next-generation sequencing platforms in systems such as genotyping-by-sequencing (Elshire *et al.*, 2011) may offer the opportunity to cost-effectively and with great versatility generate large marker panels in *T. repens*. Critical in ongoing genomic development will be genome sequence of white clover and progenitor species, which again may be enabled by information derived from genotyping-by-sequencing to aid in physical map leading to genome assembly (Mayer *et al.*, 2012).

D. Red Clover

The development of genomic resources in *Trifolium*, including red clover, and its application in forage breeding programs have recently been reviewed (Ravagnani *et al.*, 2012). Recent progress in QTL mapping in red clover is also described in several other reports (Isobe *et al.*, 2003; Sato *et al.*, 2005; Herrmann *et al.*, 2006). The first red clover linkage map contained 158 RFLP loci (Isobe *et al.*, 2003). Subsequently, a high-density map with 1,399 markers consisting of mainly SSRs and RFLPs was published (Sato *et al.*, 2005). More recently there has been significant effort on generating an integrated map that consists

of 1804 markers distributed over seven linkage groups with a total length of 836 cM (Isobe *et al.*, 2009).

Many forage crops including red clover have lagged behind other crops in the availability of genomics data. This is rapidly changing in red clover (Ravagnani *et al.*, 2012) as bacterial artificial chromosome (BAC) libraries have facilitated a physical mapping project to compare the genome of red clover with the model legume *M. truncatula*, a development that will help in understanding the genetics and genomics of red clover and to facilitate new ways of breeding new improved varieties. A number of SNP markers from the available EST database and more BAC end sequences are available in the public database (Yu *et al.*, 2010). As with the other species that are the focus of this review, the development of next-generation sequencing technology has opened up further avenues for utilizing these new genomic tools among a range of forage and grain legumes (Ravagnani *et al.*, 2012) including red clover.

VI. INNOVATIVE SELECTION STRATEGIES

A. Cultivar Types

The species considered in this review are outcrossing and typically suffer sufficient inbreeding depression to preclude the development of inbred lines. As a consequence, they are largely commercialized as synthetic cultivars, which are heterogenous populations composed of heterozygous genotypes. Commercial seed is produced by several generations of open-pollination after selection of the progenitor genotypes. The term “synthetic” is sometimes reserved for cultivars produced from parents that were selected based on combining ability and progeny testing, while designating as “open pollinated cultivars” those resulting from mass selection (Allard, 1960). However, the two types of cultivars are commonly used interchangeably and “synthetic” is often used to denote any type of open-pollinated cultivars.

In addition to phenotypic selection within a population, a related method of cultivar development is the strain cross, in which a number of genotypes from two or more populations (or cultivars) are intercrossed, followed by open-pollination of the hybrid progeny. The population resulting from a strain cross may be a candidate cultivar or it could undergo recurrent selection to further concentrate desirable alleles derived from each parental population.

Synthetic cultivars can be generated from any number of parental genotypes, with a broad distinction made between narrow-based synthetics with fewer than ten parents and broad-based synthetics having more than 50. In the forage legumes, broad based synthetics, often simply the result of mass selection for disease resistance, grazing tolerance, or other traits, have been common in the past, with less emphasis on selecting fewer genotypes based on progeny testing and combining ability to constitute a narrow-based synthetic. Broad-based synthetics have two advantages – they facilitate the development of multiple pest and disease resistant cultivars through many cycles of recurrent selection, and they minimize the potential

cost of inbreeding depression (particularly for yield) that could occur during cycles of seed increase. Such depression might be sizeable when using fewer than 7-8 parents not only in diploid (or allotetraploid) species but also in tetraploid alfalfa, where inbreeding can be emphasized by the frequent occurrence of selfing rates around or over 20% (Busbice, 1969; Riday et al., 2013). The disadvantage of broad-based synthetics, of course, is the limited ability to capitalize on and concentrate desirable alleles while excluding undesirable alleles (a limitation which may have concurred to the modest yield gains reported in Section II).

A number of selection schemes have been proposed for developing synthetic varieties of forage legumes, particularly for alfalfa. They imply the selection of parent material based on clonal evaluation, half-sib or full-sib progenies, or selfed progenies with varying numbers of selfing generations. In addition, selection may be performed among parental clones or within progeny families. Selecting and recombining partially inbred genotypes could increase the accumulation of favorable alleles with additive genetic variation, reduce susceptibility to inbreeding, reveal new genetic variation by uncovering recessive alleles, and promote greater morphological homogeneity (Rotili et al., 1994). Two selfing generations were reportedly more useful than one or three (Rotili, 1976). Few empirical comparisons have been conducted among selection schemes, and theoretical comparisons (Busbice, 1970; Casler and Brummer, 2008) indicate that different values for relevant genetic parameters would likely lead to different methods being most effective. Thus, further investigation of breeding schemes is warranted for alfalfa and the other forage legumes.

Open-pollinated cultivars make little use of non-additive genetic variation, except for narrowly-based synthetic cultivars derived from parents selected based on their pairwise hybrid progeny performance (but the portion of heterosis captured in the first hybridization decreases over cycles of seed multiplication [Gallais, 2003]). Although the genetic basis of most agronomically important traits in the forage legumes is not known, by inference from other crops and experience, we can assume that most are quantitatively inherited, with some simply inherited traits like disease or insect resistances. The facility with which most traits can be improved through phenotypic recurrent selection suggests that additive genetic variance is of primary importance although, at least for alfalfa forage yield, non-additive genetic variance is also important (Bingham et al., 1994). Heterosis for yield has been documented in alfalfa and white clover (Michaelson-Yeates et al., 1997; see also Section IV). Therefore, interest in developing and commercializing hybrid cultivars has been discussed for some time (Tysdal and Kiesselbach, 1944).

The first hybrid alfalfa varieties, which exploit a patented male sterility system to attain about 75% hybrid plants (Sun et al., 2004), have recently entered the U.S. seed market (Veronesi et al., 2010), but their superiority over conventional synthetic cultivars is unclear. Systems to produce hybrids using self-incompatibility are possible in clovers (Riday and Krohn, 2010a). Hybrids in the forage legumes are unlikely to be based

on inbred lines, at least not initially, and consequently, the cultivars would represent a mixture of genotypes, rather than the uniform genetics of typical hybrids in other crops. Interestingly, if alfalfa inbreds were developed, heterosis would not be maximized until the double cross-generation due to progressive heterosis in autotetraploids (Groose et al., 1989).

An alternative avenue to single- or double-cross hybrids is represented by semi-hybrid (or chance-hybrid) material, where hybridity between narrowly-based populations reaches 50% (Brummer, 1999; Scotti and Brummer, 2010). There is evidence for heterosis in crosses between contrasting fall dormancy types (Bhandari et al., 2007). In particular, South American populations have been shown to be a useful heterotic source for crosses with relatively dormant germplasm (Segovia-Lerma et al., 2004; Al Lawati et al., 2010), with little evidence of heterosis within surveys of more restricted sets of germplasm, even when crossed between dormancy classes (Şakiroğlu and Brummer, 2007).

Use of *falcata* and *sativa* subspecies as natural heterotic sources has been extensively evaluated, with evidence that heterosis exists (Riday and Brummer, 2002a) and may be a potentially useful source. Further evaluation of other traits in these crosses revealed few other cases of high parent heterosis for critical agronomic traits, indicating further improvement of the *falcata* source would be needed (Riday and Brummer, 2002b). This research was extended to hay production systems, revealing that the slower regrowth potential of *falcata* types was not adapted to intensive production and therefore not currently a useful source for heterosis (Riday and Brummer, 2004). Evaluation of persistence in these crosses have also shown that *falcata* persistence needs to be improved (Riday and Brummer, 2006). However, research in Australia has highlighted the potential for *falcata* germplasm to improve yield, including detection of substantial specific combining ability effects in Queensland test environments (Mackie et al., 2005). Semi-hybrid varieties issued from *sativa* heterotic sources are more likely to satisfy DUS requirements (particularly distinctness and uniformity) set by UPOV for variety registration than *sativa* × *falcata* hybrids, justifying research efforts within *sativa* germplasm. Further research is warranted to identify the genetic basis of yield and yield heterosis, including application of markers in genomic selection.

Heterotic effects have also been observed for parents derived from *M. sativa* × *M. arborea* hybridization testcrossed to other non-dormant *sativa* germplasm (Irwin et al., 2010). The possibilities of using this or other distantly related germplasm for yield improvement needs more investigation and validation, but appears promising.

B. Methods and Scope for Marker-Assisted Selection

Markers can be applied to selection in the forage legumes in several ways. For marker-assisted selection, they can be broadly grouped into those identifying individual genes or quantitative trait loci (QTL), which are then manipulated individually, and those that do not identify individual QTL but rather focus on

estimating breeding values of multiple marker alleles. In the first case, the goal is to introduce and select those specific alleles at the loci of interest. In the second case, the goal is to improve the genetic merit of a population regardless of which loci are selected. In other words, while the former method is targeting specific alleles of specific genes, which are assembled machine-like into desirable genotypes, the latter is a “black box” approach where various genes and alleles contribute in different combinations to the genetic merit of different individuals, so that a desirable individual genotype may vary considerably from another equally desirable genotype in terms of alleles that it carries.

In all cases, these marker applications depend on linkage disequilibrium (LD) between a marker locus and a gene to be effective. In the best case, the marker itself is the cause of allelic variation – that is, it is a functional marker (Andersen and Lübberstedt, 2003). However, in most cases, the marker is not exactly at the causative polymorphism. Therefore, recombination can occur between the marker locus and the trait locus. The amount of recombination depends on the amount of linkage disequilibrium, and the less disequilibrium, the weaker the ability to identify markers linked to traits or to use the linked marker to make improvement. Conversely, if LD is extensive, such as that found in an F_1 population derived from heterozygous parents, a common structure in the forage legumes, then markers could be identified that are a significant distance from the trait locus. In these cases, a more appropriate population to target a trait locus may be an association mapping population in which historical recombination has limited LD to some extent. An association mapping population could be a collection of germplasm resources or a breeding population.

1. Biparental populations

Most obviously, markers can be used to tag a desirable QTL or gene (allele, actually) using a population derived from a biparental cross (or even the selfed progeny of a heterozygous individual). This classical QTL mapping method is mainly useful in a breeding sense if one of the parents has a desirable allele to be introgressed – mapping can be done to identify the locus and markers associated with it, which can then be used to incorporate the new allele into breeding populations. The primary limitation of this method is that resolution of marker-trait associations is typically poor, and consequently, decoupling of the desirable marker and trait alleles is possible through cycles of selection and recombination. The second limitation of biparental populations is that only genes segregating in this population will be mapped, and if multiple other loci are important on a population-wide basis, they will be missed. The combined study of several biparental mapping populations, especially those connected by one or more common parents, can alleviate this problem.

In alfalfa, QTL have been mapped for numerous traits, including yield (Musial *et al.*, 2006; Robins *et al.*, 2007b), plant height and regrowth (Robins *et al.*, 2007a), persistence (Robins *et al.*, 2008), self-fertility (Robins and Brummer, 2010), winter-

hardiness (Brouwer *et al.*, 2000), and resistance to *Stagonospora meliloti* (Musial *et al.*, 2007), *Colletotrichum trifolii* (Mackie *et al.*, 2007), and *Phytophthora medicaginis* (Musial *et al.*, 2005). Persistence has also been mapped in red clover (Herrmann *et al.*, 2008; Klimenko *et al.*, 2010). In white clover, QTL discovery for traits has included spaced plant morphogenesis in an F_2 inbred population in the absence of grass competition and grazing (Cogan *et al.*, 2006), seed yield on spaced plants (Barrett *et al.*, 2005b), salt tolerance (Wang *et al.*, 2010), and stolon diameter, stolon number, and internode length (Zhang *et al.*, 2010). White clover full-sib pair cross mapping populations have been assessed for aerial and root morphological characters (Jahufer *et al.*, 2008; Jahufer *et al.*, 2013) but have not yet had QTL discovery reported. Qualitative trait maps have also been developed (Barrett *et al.*, 2005b; Casey *et al.*, 2010; Tashiro *et al.*, 2010; Wang *et al.*, 2010), identifying independent loci controlling leaf colour, self-compatibility, and in the related species *T. semipilosum* a single gene resistance to root nematode infection.

Despite these QTL studies, there are few published examples of marker-assisted selection in multi-parent forage legume breeding populations. One exception built on the early QTL discovery for seed yield (Barrett *et al.*, 2005b) used the SSRs subtending the QTL to type alleles in breeding populations, identifying significant associations. On the basis of single marker SSR assays with variable allele composition, phase and frequency, associations with a mean 40% differential in seed yield were detected in eight out of twelve populations surveyed (Barrett *et al.*, 2009). These seed yield markers are now used in white clover breeding programs in New Zealand.

2. Association mapping

Association mapping can avoid the limitations of biparental populations by evaluating a broader diversity of germplasm and populations that have experienced more recombination, and hence, have a lower extent of LD. Generating an association mapping population by intercrossing agronomically valuable populations that contrast for molecular marker diversity, geographic origin, and/or phenotype can widen the inference space and applicability of the marker assessment. Appropriately constructed populations can also help dissect major genotype \times cropping environment interactions that may occur across the target cropping environments, provided suitable multi-environment phenotyping is conducted. Conversely, sufficient marker number (and the relative costs) can be an issue for association mapping, particularly in highly diverse populations where LD will be limited. For example, a minimum of 1,000 markers were needed to fully explore the genome of one alfalfa breeding population that was used in an association mapping experiment (Li *et al.*, 2011).

Few examples of association mapping have been published in the forage legumes, which likely reflects the general lack of significant numbers of markers to saturate the genome under conditions of short LD. In alfalfa, a breeding population was

assessed with a limited number of SSR markers, and while this was insufficient to cover the entire genome, yield QTL were identified, one of which was also identified in a previous experiment with a biparental population (Li *et al.*, 2011). Two experiments have evaluated sequence variation in candidate genes for association with traits: flowering time and stem height were related to variation in the *CONSTANS* gene within alfalfa cultivars (Herrmann *et al.*, 2010), but sequence variation in several lignin biosynthesis genes was not related to lignin content or cell wall composition in a broad collection of diploid alfalfa germplasm (Şakiroğlu *et al.*, 2012).

Effective association mapping will be facilitated by the development of SNP markers (Han *et al.*, 2011; Li *et al.*, 2012) (see Section V). The SNP genotyping costs can be high for single SNP platforms, such as high resolution mapping (HRM; Han *et al.*, 2011). Costs can be reduced 10–20 fold by using SNP array technologies such as an Infinium array (Li *et al.*, 2014), but these typically require large numbers of samples to be analyzed simultaneously, which may be beyond the budgets of most forage legume experiments. The development of methods to genotype directly from sequence data, such as genotyping-by-sequencing (GBS) (Elshire *et al.*, 2011) and RAD-tags (Baird *et al.*, 2008), can further reduce the costs per SNP data point, perhaps by half. However, these methods have drawbacks discussed later on in terms of missing data and difficulties of alignment, until genome sequences are available for each individual species. Still, in recent experiments we have identified over 4,000 GBS-generated SNP markers aligned to *M. truncatula* in two alfalfa populations (Annicchiarico, Brummer *et al.*, unpublished data). Besides, these limitations will be overcome by higher sequencing capabilities for a given cost and by the development of whole-genome sequences for all the major forage legumes, with projects already underway for alfalfa and white clover.

3. Bulk segregant analysis

The concept of bulk segregant analysis (BSA) (Michelmore *et al.*, 1991) has not been widely used in forage legumes. As originally proposed, BSA involves plants from a single population contrasting in phenotypes (e.g., disease resistant vs. susceptible). However, a conceptually similar method is to develop bulks of genotypes from different cycles of selection. The bulked samples can be evaluated with markers to identify differences; generally, differences observed in marker allele patterns are related to the gene(s) under selection. This procedure has been used effectively to identify markers linked to freezing tolerance in alfalfa (Castonguay *et al.*, 2010; Rémus-Borel *et al.*, 2010). These markers identified polymorphisms in a dehydrin gene and were used successfully to select indirectly for freezing tolerance. In a similar manner, a putative locus controlling resistance to whiteflies in alfalfa has recently been identified using SNP markers generated with GBS (Brummer, Monteros *et al.*, unpublished data). Also BSA, like association mapping, requires high density of markers, in order to cope with short LD.

4. Candidate gene methods

Many genes involved in biosynthetic pathways, such as flowering date (Putterill *et al.*, 2004; Oh and Lee, 2007; Jung and Müller, 2009; Jarillo and Piñeiro, 2011), lignin biosynthesis (Baucher *et al.*, 1998; Boerjan *et al.*, 2003; Boudet *et al.*, 2003), branching (Leyser, 2003; McSteen and Leyser, 2005; Beveridge, 2006), symbiosis (Gibson *et al.*, 2008), have been identified from genetic, transcriptomic, mutation, transgenic experiments in many species (see <http://medicago.jcvi.org/medicago/> for the version 4.0 of the *M. truncatula* genome sequence). Gene function is often known or inferred based on RNA sequencing, such as the gene expression atlas developed in *M. truncatula* by evaluating various organs at different developmental stages (Benedito *et al.*, 2008), particularly for genes related to symbiosis (El Yahyaoui *et al.*, 2004). Because most biosynthetic pathways are common to the plant kingdom, genes with similar sequences likely have similar functions, especially for species in close phylogenetic proximity (e.g., Hecht *et al.*, 2005 as an example for flowering genes). Analysis of phenotypic mutants (Tadege *et al.*, 2008; Le Signor *et al.*, 2009) can lead to the identification of the gene that is responsible for the trait variation and potentially to all the genes of a pathway, not only those that explain natural variation. Dissection of the genetic control of agronomic traits in the forage legumes through gene expression profiling, metabolomics or proteomic analysis, or mutant screening has not been widely done as in model systems, but information accumulated on other species can be usefully applied to alfalfa and the clovers.

Candidate genes can be located by identifying a QTL, and then fine mapping the underlying gene(s) to a small genomic region. In *M. truncatula* or other fully sequenced species, all the genes in this region can be identified (e.g., <http://medicago.jcvi.org/medicago/>). In forage legume species without a full genome sequence, alignment of a QTL region to *M. truncatula* through markers can give a reasonable location to search for genes. These genes become candidates to explain the variation for the trait.

In general, the effect of every possible candidate gene (i.e., from a pathway or from a QTL region) on a phenotypic trait cannot be evaluated. The objective is to find the genes involved in variation for a trait, not all the genes that contribute to the trait. Some genes in a biosynthetic pathway, for example, are not interesting from a breeding perspective because any mutation has dramatic negative effect on the trait. A focused list of candidate genes can be developed by examining commonalities from the literature, expression or mutant analyses, and QTL studies (Pflieger *et al.*, 2001). By using this method, the list of 572 genes included in a QTL region involved in flowering date in *M. truncatula* was reduced to six by only selecting genes known to belong to the flowering pathway (Pierre *et al.*, 2008). The comparative gene expression of the two parents of a mapping population indicated that one of these six genes was a candidate to explain differences in flowering date (Pierre *et al.*, 2011).

Confirming the phenotypic effect of a candidate gene can be done through association mapping based on a candidate gene or through bulk segregant analysis. As described above, in association mapping the sequence polymorphism of the gene will be compared to phenotypic variation in a well-chosen population. In a bulk segregant analysis, the allelic variation of the gene is compared in two divergent populations established after selection on a phenotypic trait. These two methods give information on the gene function and the identification of positive alleles, which can be directly used in breeding programs. An association mapping study based on candidate genes confirmed a flowering time gene was involved with stem height (Herrmann *et al.*, 2010), and selection made on alleles of this gene resulted in contrasting populations for stem height (Julier, 2012). In populations coming from selection for frost tolerance, the frequency of dehydrin variants was different, showing the importance of dehydrin genes in frost tolerance in alfalfa (Dubé *et al.*, 2013).

Finally, cloning a candidate gene and down- or up-regulating it in transgenic plants is perhaps the best confirmation of the gene's effect. Genes from the lignin pathway were under-expressed to test their effect on lignin content and composition in alfalfa (Guo *et al.*, 2001a; Guo *et al.*, 2001b). A gene identified as being involved in anthracnose resistance in *M. truncatula* conferred resistance to initially susceptible alfalfa genotypes (Yang *et al.*, 2007; Yang *et al.*, 2008).

5. Genomic selection

Genomic selection (GS) (also known as “whole-genome selection”) (Heffner *et al.*, 2009; Jannink *et al.*, 2010; Lorenz *et al.*, 2011; Hayes *et al.*, 2013) is based on the premise that markers saturating the entire genome can reliably provide an estimate, in aggregate, of the breeding value of an individual plant. By using an initial “training population” of plants, phenotypes and genotypes are both evaluated and an estimated breeding value for each marker is determined. The sum of marker breeding values for a given individual could then be estimated, even in the absence of phenotypic data, and consequently, the model based on the training population can be applied to a breeding population so that selection could be made simply on the genomic estimated breeding values of each individual.

The critical component of GS is the generation of many markers to saturate the genome. Until recently, this has not been possible in the forage legumes. Large numbers of SNP data can now be produced through recent sequencing technologies such as GBS (Elshire *et al.*, 2011) and RAD-tags (Baird *et al.*, 2008). De novo assembly of the short DNA sequences generated by these methods leads directly to the assignment of SNP genotypes to individuals. The primary limitation of these methods are a large amount of missing data, where some individuals are not sequenced for particular loci, or when the number of sequencing reads for particular loci is too low (for instance, to be able to say, with confidence, that the genotype is homozygous or heterozygous). Additional complexity attends tetraploids. For autotetraploids like alfalfa, the allele dosage cannot be determined

without a large number of reads per locus; for allotetraploids like white clover, discriminating between heterozygosity at a locus and contrasting sequences at homoeologous loci can be problematic.

In forage crops, the most obvious way to set up a GS program would be within the context of an intra-population recurrent selection program, whereby a set of genotypes would be evaluated with genome-wide markers and phenotyped as clones or progenies for the trait(s) of interest. The GS model developed based on this initial cycle of selection would then be applied to the resulting population, through several cycles conducted in the glasshouse in which seedlings were genotyped and selections made based solely on GS-predicted breeding values (Li and Brummer, 2012). However, phenotypic evaluations would partly continue in the field, to generate additional information to improve the GS model. The actual advantage of including GS in forage legume breeding programs is still unknown, depending on the balance of costs (deriving from additional genotyping cost and reduced phenotyping costs, if any) and the possible increase in the rate of genetic gain per unit time that it can provide (as discussed later on).

6. Other uses of markers

Outside of QTL detection and location and genomic selection, DNA markers can be meaningfully applied to breeding programs in other ways. For instance, using markers to infer paternity could substantially increase genetic gain, simply by controlling both parents of selected plants and not just the maternal parent, as is often done (Riday, 2011).

Markers have also been used to try to predict heterosis, with evidence for a relationship between marker-based diversity and heterosis of alfalfa parent material (Annicchiarico *et al.*, 2010; Bhandari *et al.*, 2011). The usefulness of marker-based diversity for predicting forage yield of alfalfa synthetic varieties is controversial. Yield was not correlated with marker diversity among 93 potential parents (Kidwell *et al.*, 1999), whereas another study has shown a relationship to exist (Tucak *et al.*, 2011).

Markers can also be used to assess germplasm diversity and population structure, which could assist breeders with identifying underutilized germplasm for a breeding program, or with understanding how selection progress may be affected by underlying structure. For example, the diversity of diploid alfalfa germplasm has been recently elucidated using SSR markers (Şakiroğlu *et al.*, 2010), which could help identify desirable phenotypes that have different marker profiles (Şakiroğlu *et al.*, 2011). Marker-based diversity among germplasm sources may only limitedly infer their morpho-physiological diversity, based on reports relative to red clover (Dias *et al.*, 2008; Pagnotta *et al.*, 2011) and white clover (Kölliker *et al.*, 2001b; Annicchiarico and Carelli, 2014).

Markers can also be useful for cultivar identification (Kölliker *et al.*, 2001b), the investigation of phylogenetic relationships across species (Ellison *et al.*, 2006) or within species (Annicchiarico and Carelli, 2014), and the assessment of cross

pollination rates. For example, in alfalfa they have revealed relatively high rates of self pollination which may contribute to yield depression through inbreeding or lower than expected hybridity in hybrid populations (Gjuric and Smith Jr, 1996; Riday *et al.*, 2013).

C. Integration of Field-Based and Marker-Assisted Selection

Marker tools by themselves are not much use unless integrated meaningfully into a breeding program. Marker-based selection schemes still need to consider the same standard issues of all breeding programs, namely, the clear specification of the target environments in which the resulting cultivar will be grown, the ability to accurately measure the traits of interest, and the need for phenotypic assessments to be made under conditions as close as possible to those conducted by the farmer in the target environments.

The predicted gain (ΔG) arising from one cycle of phenotypic selection for yield in a given genetic base and selection environment can be expressed as (Falconer, 1989):

$$\Delta G = i h r_g s_A$$

where i is the standardized selection differential, h is the square root of narrow-sense heritability in the selection conditions, r_g is the genetic correlation for genotype yield responses between selection and target conditions, and s_A is the standard deviation of breeding values. Optimizing the phenotyping conditions to reflect the target growth conditions is crucially important in order to maximize r_g , and this is especially true in the case of yield. For example, the phenotyping of space planted material, or that of material in pure stand while targeting mixed stand conditions, would each reduce by over one-third the predicted genetic gain, according to data in Table 4. Phenotyping under favorable moisture conditions while targeting drought-prone conditions, or phenotyping mown genotypes while targeting severe grazing conditions, may lead to essentially no gain (Table 4).

When using a genomic selection model, ΔG can be expressed by a similar formula:

$$\Delta G = i r_A r_g s_A$$

where r_g is the genetic correlation for genotype yield responses between phenotyping and target conditions of crop utilization, and r_A is the genomic selection accuracy, i.e., the correlation between predicted and true breeding values in the phenotyping conditions, which substitutes for the term h of the phenotypic selection formula. While the comparison of r_A in GS vs. h in phenotypic selection is relative to yield gains per selection cycle of the two selection strategies, the comparison of yield gains per unit of time takes account of the higher number of selection cycles that genomic selection can ensure relative to phenotypic selection. An additional term indicating the relationship between marker breeding values in the training population and in the pop-

ulation undergoing selection, particularly if the two populations do not belong to the same genetic base, could be added in the latter formula if it could be determined; in practice, this term effectively is confounded with r_A . To what extent a GS model developed in one population can be used effectively in another population is a question of considerable practical interest for breeding programs.

Genomic selection (or other marker-assisted selection methods) do not eliminate the crucial need for the phenotyping environment to reflect the target environment. The low r_g in the latter formula implies low yield gains even if the marker accuracy for the phenotyping environment (r_A) is very good. Overall, the practical usefulness of genomic selection models and marker-assisted selection procedures for complex quantitative traits (e.g., forage yield, or tolerance to drought) depends on how well the germplasm and phenotyping environments used for marker-trait association or GS model development reflect the breeding population and environments to which they are targeted. Opportunities for genomic selection do not free breeding programs from the need to define suitable adaptation strategies, e.g., whether selecting for wide or specific adaptation to rainfed and irrigated environments within a target region (e.g., Annicchiarico, 2007c), because genotype yield responses predicted from useful markers and genes can be expected to parallel those observed for yield itself. Marker information, however, can support decisions and implementation of optimal selection strategies (e.g., limiting the costs of multi-environment testing when breeding for wide adaptation; or facilitating the early allocation of genotypes to specifically-adapted germplasm pools).

Compared with phenotypic selection, genomic selection allows for more selection cycles and therefore accumulated selection gains and possibly higher selection intensity (depending on the evaluation cost per genotype of each selection procedure), even though the r_A term might compare unfavorably to h . We have preliminary evidence in alfalfa biomass yield that accuracy of GS can exceed 0.40 for the purpose of clonal selection (Li, Brummer *et al.*, unpublished data). In most cases, however, the relevant r_A value in the formula above is relative to GS accuracy for parent selection based on half-sib performance. The values of h for alfalfa forage yield are likely in the range of 0.40–0.55 (as inferred from narrow-sense heritability estimates summarized in Section IV). Preliminary results suggest that r_A values are lower than h but high enough to provide comparable or somewhat higher expected yield gain for GS relative to phenotypic selection, if the frequency per unit time of genomic selection cycles is at least twice that of phenotypic selection cycles (Annicchiarico, Brummer *et al.*, unpublished data).

The use of markers in forage legume improvement programs has not been as rapid as expected 20 years ago, when the first alfalfa genetic linkage maps were published (Brummer *et al.*, 1993; Kiss *et al.*, 1993). Although this can be blamed on the lack of markers, few researchers, and other similar issues, perhaps a greater reason is that breeding programs as currently constructed are not ideally suited to integration of markers. Biparental

mapping populations are usually unavailable in breeding programs – they are usually constructed from contrasting parental genotypes ill suited for highly advanced agronomic performance. In any case, phenotyping a mapping population would be expensive, and selection within the population could pose a risk of inbreeding depression in a synthetic cultivar. QTL detection in multiple biparental populations (Blanc *et al.*, 2006; Pauly *et al.*, 2012) could be exploited for marker-assisted selection in a breeding program, if the progeny of a polycross between a small number of parents was considered (P. Barre, pers. comm.). QTL detection through association mapping or bulk segregant analysis may not be useful for breeding populations which are genetically distant from the mapping population, although this should be verified and is likely to depend on the trait being considered (an alien introgression may be highly suited across all cultivated populations, for example).

Constructing narrowly-based breeding populations from few founding genotypes and recurrently selecting within them would lead to longer chromosome segments in LD that could be easily saturated with markers. In contrast, the full genomic characterization of broadly-based, diverse germplasm pools with short LD is not possible even with GBS markers at the current time, but evaluating broad germplasm pools would enable a single GS model to be applied for different traits throughout the entire genetic base of the breeding program, and could also afford a greater ability to satisfy the distinctness requirement for various candidate varieties which were recurrently selected from the same population. Thus, defining the population structure and extent of LD remains a crucial issue for the successful application of marker-assisted selection.

Considering the potential of GS leads us to the conclusion that we need to attend to several issues in all our breeding programs in order to realize the improved gain possible with markers. In particular:

- (i) evaluating phenotypes throughout the main target environments, choosing locations based on the pattern of genotype \times environment interaction in the target region defined by state of the art genotype modeling and environment classification techniques (Annicchiarico, 2009);
- (ii) evaluating phenotypes of parental genotypes based on sward plots of half-sib or selfed progenies rather than as spaced plants (as usually adopted for cloned genotypes), to closely represent actual dense planted production environments (evaluating half-sib or selfed progeny seed rather than clonal material also facilitates the preservation of mapping/reference populations and the implementation of multi-environment experiments);
- (iii) using marker information with some specificity depending on the target cultivar to be developed. For example, separate GS models could be developed for yield selection in a specific environment and for wide adaptation across different cropping systems. The selection of traits of specific interest for a subset of target environments could rely on culling levels for markers associated with these traits (or, alternatively, based on a selection index applied in the GS model). This is exemplified in Figure 5 for the hypothetical case of alfalfa breeding for a region requiring low winter-dormant varieties (as the southern United States, or Mediterranean-climate regions), generating four varieties targeted to different cropping conditions from parents selected on the basis of forage yield breeding values estimated by one or two genomic selection models and the possession of sets of markers that are useful for the target cropping conditions. The development of a single cultivar equally adapted to and productive in all environments, or of one carrying all desirable traits, is unlikely, but developing many cultivars for a plethora of environments is problematic from a marketing standpoint. Therefore, a balance needs to be struck so that enough cultivars adapted to a range of environments is developed.

Marker-based selection procedures will be limited to a specific genetic base. Whether this poses a problem to a given breeding program is not known at the outset. As anticipated, if the initial training population is drawn from a widely-diversified reference genetic base, the GS model developed could be broadly applicable, but whether such a model could be developed needs empirical testing. The problem of short LD implied by this strategy could be compensated by high marker numbers made available by GBS or similar methods. The number of markers and their cost need to be assessed, however, but one may expect costs to decline significantly in the near term.

The limitation of GS model development is in the numbers of genotypes for which accurate and robust phenotypic data can be acquired. In general, one may conceptualize a breeding program including a GS component in the following way.

- (i) The overall reference population. A broad collection of germplasm serves as the base of the overall program. This may be a series of cultivars and advanced breeding populations belonging to the breeder together with additional germplasm, landraces, or cultivars he/she has accumulated over time to fit particular needs of the program. This overall collection is evaluated in simple ways – e.g., spaced plant nurseries – to identify plants with merit and to remove the considerable amounts of material with limited value. These nurseries could be on the order of several thousands of plants, probably too many to be usefully screened with markers (unless specific traits could be targeted).
- (ii) The elite reference population. A more elite collection of breeding populations and genotypes derived from the larger pool, which serves as the primary source of parents for new cultivars. These populations and genotypes may be the targets of GS. A GS model that could be applicable to this set of elite germplasm may be feasible, but perhaps even smaller subsets destined for particular target

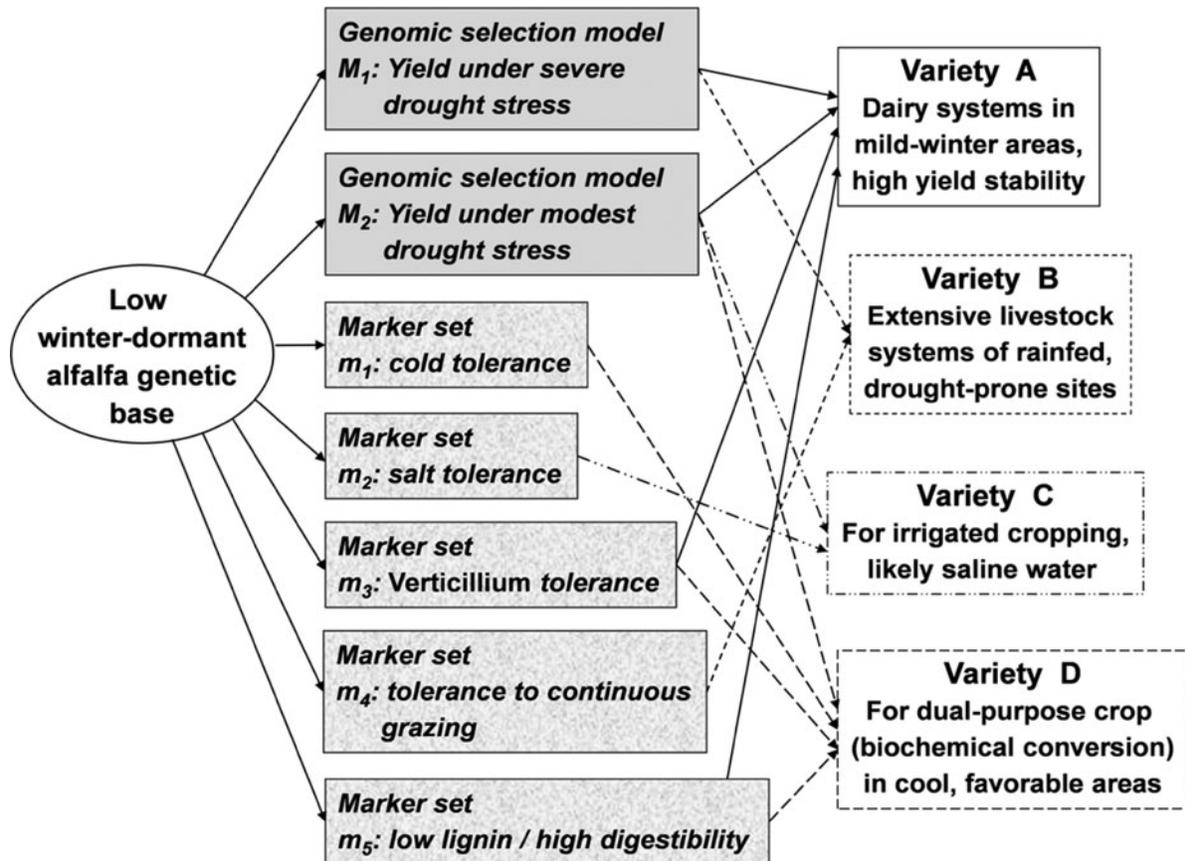


FIG. 5. Hypothetical exploitation of marker information in breeding low winter-dormant alfalfa varieties for different cropping conditions within a target region.

environments with particular target traits is needed. In any case, the GS model will require good phenotypic data on a training population, with periodical updating of the model by additional phenotyping of members of the reference population. A set of genotypes from the elite reference population will need to be genotyped and phenotyped. This set cannot be too large particularly if the goal is to test these in mini-sward plots where seed quantities will be limited, but if it is too small, the GS model will not be robust. We are interested in estimating marker allele breeding values, so the level of replication is the allele, not the genotype; therefore, emphasis may be placed on evaluating more families in fewer replications within test locations.

- (iii) The elite base could be regularly reinforced with additions from the overall population. Small changes to the elite genetic base would (likely) not justify changes in marker-assisted selection procedures for genetically complex traits, provided that the newly-added germplasm is a fraction of the total genetic base and is intercrossed with it before selection. However, occasional substantial changes to the elite genetic base may be needed with some periodicity (e.g., every 10 or 15 years), to prevent breeding progress for major traits from being seriously hampered by fixation

of major favorable quantitative alleles existing in the reference genetic base, as well as to help breeding programs subjected to DUS obligations in breeding sufficiently distinct material for variety registration. Each such change would require the redefinition of marker-based procedures for complex polygenic traits through new extensive study.

D. Other Indirect Selection Strategies

1. *In vitro* selection

Defining and applying *in vitro* selection procedures for tolerance to abiotic or biotic stresses can make selection more rigorous and predictable. However, their use on forage legumes has been very limited so far (Tapingkae *et al.*, 2012). The poor *in vitro* regeneration of these species hinders the application of the complete set of *in vitro* selection stages, which includes variant or mutant induction, *in vitro* selection, and regeneration of best plant material. However, *in vitro* mass selection performed on seedlings may provide a cost-efficient means for selecting for tolerance to specific stresses. An *in vitro* protocol has recently been established to screen alfalfa for aluminium tolerance (Khu *et al.*, 2012), and other protocols may be successful for other target traits, e.g., salt tolerance (Safarnejad *et al.*, 1996).

2. Artificial environments and selection/phenotyping platforms

The selection of complex traits to improve performance under different climatic conditions could be done with artificial environments and phenotyping platforms, to evaluate and select desirable genotypes under highly controlled conditions. New phenotyping facilities, such as LemnaTec and others, could potentially reduce evaluation costs and improve genetic gain compared to field testing, if their results were sufficiently consistent with field-based ones. If reliable, such facilities can allow for much better control of environmental conditions (e.g., for the level of drought stress, or low temperatures) and their homogeneity across the evaluated material in comparison with field testing.

Carefully chosen artificial environments can greatly facilitate the implementation of cost-efficient, ecologically-based breeding strategies. For example, two environmental factors, i.e., summer drought stress (limited under irrigated cropping; high under rainfed cropping) and soil type (sandy-loam or silty-clay), associated with large alfalfa cultivar \times location interaction for forage yield across northern Italy (Annicchiarico, 1992), were exploited to set up four artificial environments created by the factorial combination of these factors in one selection location. These artificial environments successfully reproduced the observed cultivar responses and the expected responses of local landraces across the target region (Annicchiarico and Piano, 2005), and offered the opportunity for inexpensive selection for specific or wide adaptation (Annicchiarico, 2007c). Phenotyping platforms including rain-out shelters, or drought bins within glasshouses, are increasingly used for characterizing the drought tolerance of plant material.

Various experimental devices and procedures have been developed to successfully screen for other abiotic or biotic stresses (see also Section IV). For example, Castonguay *et al.* (2009) have devised a screening methodology for the reliable identification of alfalfa genotypes with superior tolerance to freezing. The assessment of populations recurrently selected using this approach revealed significant increases in cold tolerance from -25°C to -29°C lethal temperature for 50% of the plants after six selection cycles. A cold tolerance screening test developed in UK for white clover showed good consistency with, and better ability to discriminate cold tolerant material than, field testing (Annicchiarico *et al.*, 2001).

Flowing solution culture systems have been used to analyse the response of breeding material to nutrient availability. Recent studies on a white clover mapping family, using such a facility, have shown significant genetic variation in phosphorous uptake and utilization (Marshall, unpublished data) with QTL identified for this trait. More recent developments include the application of large scale phenomics capability such as The National Plant Phenomics Centre (NPPC). This new BBSRC-funded facility, based within IBERS, provides automatic, high throughput, non-destructive developmental and physiological imaging of shoots and roots of plants automatically moved from controlled envi-

ronments to imaging systems. Simpler tools for root phenotyping have also been developed in each institution represented by the authors.

Increasing effort in the development of high-throughput phenotyping equipment and methodologies is justified. Such improvements would overcome the limitation on the number of genotypes that can be tested and improve the standardization of selection conditions. Although increasingly reliable, such facilities will not substitute for final testing under field conditions.

3. Physiological traits

The relationships of morphophysiological traits with major traits targeted by breeding programs has been anticipated in Section IV. Although these relationships were often meant to be exploited as indirect selection criteria for target traits, their exploitation by breeding programs has been modest. For several traits (e.g., carbon isotope discrimination), this could be attributed to relatively high observation cost, when applied to large numbers of genotypes. However, the potential usefulness even of relatively expensive traits for some breeding context, e.g., the identification of contrasting parental material for mapping populations, has probably been overlooked. Simple, inexpensive indirect selection criteria, e.g., lower leaf wilting or leaf senescence for alfalfa drought stress tolerance (Annicchiarico *et al.*, 2013) or seed yield per inflorescence for alfalfa seed yield (Bolaños-Aguilar *et al.*, 2001), remains those of practical interest for massive genotype selection.

E. Transgenic Varieties

Bringing transgenic forage crops to market is problematic for various regulatory reasons (Wang and Brummer, 2012) and may raise public concern, especially in Europe, because of the difficulty of trait confinement arising from the outbred reproductive system and the frequent presence of feral or wild populations in these species (Bagavathiannan *et al.*, 2012). Only few transgenes representing major improvements to a crop are or will be commercialized in the United States, under the current scenario of high deregulation costs (for example, an alfalfa transgene may require adoption on nearly 50% of the United States growing area to make its development and marketing profitable [M. McCaslin, Forage Genetics Int., pers. comm.]).

In alfalfa, RoundupReady cultivars, tolerant to the herbicide glyphosate, are on the market in the United States. Alfalfa with modified lignin (Guo *et al.*, 2001a) has beneficial animal nutritional properties, and commercial cultivars are currently being evaluated for deregulation in the United States (M. McCaslin, Forage Genetics Int., pers. comm.).

A third major gene for alfalfa and white clover would be one that leads to the production of condensed tannins, with the aim of increasing the fraction of by-pass protein, reducing the incidence of pasture bloat and minimizing nitrogen losses in urine. Recently, key regulatory factors in *T. arvense* have been identified that control the expression of condensed tannins

in white clover and alfalfa (Hancock *et al.*, 2012), potentially leading to significant advances for this trait.

The use of transgenics to create novel variation for key traits has been of on-going interest for over 20 years. Early work in white clover targeted primarily insect tolerance (Voisey *et al.*, 1994; McManus *et al.*, 2005) and virus resistance (Panter *et al.*, 2012), but none has moved beyond initial field trials. Numerous genes have been engineered into alfalfa, including genes to improve Al soil tolerance (Reyno *et al.*, 2013) and to enhance staygreen (Zhou *et al.*, 2011), among many others (Brummer, 2004). Despite the challenges of commercializing these genes, the transgenic process offers a proof of concept regarding gene function and phenotype expression. Subsequently, once a gene has a known function, a search for mutants using TILLING (McCallum *et al.*, 2000) or natural variants using ecoTILLING (Comai *et al.*, 2004) can be undertaken. Mutants that have the desired trait could then be used directly in a breeding program. Of potentially even greater interest are the powerful technologies of genome editing (Shukla *et al.*, 2009; Cong *et al.*, 2013) that can potentially generate desired mutations in the gene of interest. These technologies are very much in the development phase in plant genetics, but if they can avoid the challenges transgenes face for commercialization, they may play a very large role in future cultivar development.

Biotechnological techniques that require the use of *in vitro* regeneration may suffer, especially in alfalfa, from the small number and the modest agronomic value of the available genotypes with good regeneration ability (Weeks *et al.*, 2008), a problem which can be overcome through extensive screening of elite genotypes (Confalonieri and Annicchiarico, 2014).

VII. CONCLUSIONS

The increasing need for crop-livestock systems which reduce energy consumption, greenhouse gas emissions and the use of herbicides and pesticides, while increasing the protein self-sufficiency, safeguarding the agricultural value of less favorable environments and adapting to climate change, emphasizes the strategic role of perennial forage legumes. The environmental interest of perennial crops is confirmed by the on-going scientific effort of turning major small-grain cereals into perennial crops (e.g., wheat: Hayes *et al.*, 2012b).

The agronomic value of major perennial forage legumes has been greatly enhanced by plant breeding mainly for disease and pest resistance. But several largely unmodifiable biological factors hinder the genetic progress for forage yield. We believe that breeding of these crops is going to be more successful through: (i) better exploitation of primary to tertiary gene pools; (ii) improved, less expensive and/or high-throughput phenotyping and selection procedures (ranging from field, managed or artificial environments to *in vitro* assessment); and (iii) marker-assisted selection (especially through the high number of markers and low marker cost which are allowed for by genotyping-by-sequencing approaches). To be really successful, each of these assets needs to be carefully integrated into selection schemes in an

TABLE 5

Alfalfa tolerance to aluminium as expressed by lower reduction of shoot dry weight in unlimed soil relative to limed soil, obtained by transgenic approaches and by two generations of among and within family selection in unlimed soil

Approach	Shoot dry weight ratio ^a
Transgenic^b	
No transgene (control)	0.66 b
Citrate synthase	0.91 a
Proton pump	0.98 a
Both transgenes	0.95 a
Field selection^c	
Cycle 0 (control)	0.65 b
Cycle 2	0.99 a

^aMeans with different letter differ at $p < 0.05$.

^bSource: Reyno *et al.* (2013).

^cBrummer *et al.*, unpublished data.

ecological prospect capable of coping with and possibly exploiting the large genotype \times environment interactions occurring in these species with respect to climatic, soil, crop management and crop utilization conditions of the target environments.

The value of field-based selection in the target conditions of utilizations (using state-of-the-art designs and procedures for experiment error control) should not be overlooked. For example, in alfalfa two cycles of among and within family selection for tolerance to aluminium under field conditions were able to provide genetic gains comparable with those obtained through different transgenic approaches (Table 5). Innovative variety types designed for greater heterosis exploitation may offer further opportunities for forage yield improvement, although their actual advantage over the conventional synthetic variety breeding (which also requires optimization, especially for alfalfa) still awaits verification. Commercial transgenic breeding might be limited to a few breakthrough traits under the current regulations, and is likely to face public concern and technical difficulties due to the risk of gene-flow that it may imply.

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