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# Global impact of sown temperate pastures on productivity and ecosystem stability – what progress have we made?

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**Abstract.** Twenty years ago, in 1993, we published one of the first two alfalfa genetic linkage maps. At the time, hopes ran high that genetic marker technologies would revolutionize selection, making the development of superior cultivars both easier and faster. The objective of this paper is to critically examine forage improvement since that time and to suggest ways to more fully capitalize on those initial hopes in the future. Marker studies have been conducted around the world, identifying quantitative trait loci (QTL) for the major agronomically important traits, including biomass yield, nutritive value, disease resistance, abiotic stress tolerance, and others. But progress has been slow and no cultivars on the market today have been bred using marker technology in a significant way. I will discuss reasons for the limited progress, including the lack of a critical mass of researchers, funding limitations, and genetic complexities integral to the crop. Despite the limitations, I suggest that the international community can do a better job integrating resources to achieve better genetic gain in breeding programs. I will discuss focused methods that could successfully integrate markers into breeding programs by manipulating individual QTL from unadapted germplasm and by applying genomic selection to accelerate breeding cycles. Even so, the real world value of these technologies needs to be carefully considered before they can be adopted in a commercial scale.

**Keywords:** Phenotyping, molecular markers, temperate pastures.

## Introduction

Looking into the near future, three major trends face the world: (1) a more unstable and unpredictable climate; (2) a growing world population increasingly desiring a diet rich in animal products; and (3) the necessity that human activities minimize negative environmental impacts. These trends pose challenges – and opportunities – for plant breeders to develop productive cultivars that are resilient to climate change and whose cultivation improves the environment (Brummer *et al.* 2011). In this paper, I will focus on methods to improve forage crop breeding and assess how well new technologies been applied to actual cultivar development. I will then discuss possibilities for future genetic gains. I will concentrate on temperate forages, particularly alfalfa (lucerne), white clover, and the fescues/ryegrasses, over the past 20 years – representing two decades since the first DNA based molecular marker linkage maps were published in alfalfa. I apologize up front for not citing all relevant work or for inadvertently overlooking significant literature. Much good work is being done throughout the world, but it is outside the purview of this paper to make a comprehensive summarization of it all.

## What is our goal?

With the exception of the cash hay business, forage crops are not commodities. Instead, they are either directly consumed by or harvested and fed to livestock (or horses, which represent a wholly different industry, with a different economic model than the livestock industry). The ultimate selection target – and the true measure of the value of forage – is the animal product produced from a given mass

of forage or a given land area of forage production. Yet this true measure of the forage value is essentially never measured in breeding programs, which rely instead on selection of various aspects of forage nutritive value, which together with biomass yield could approximate potential meat or milk production. Because the genetic potential of forages is greatly affected by farmer management, we typically assume that the farmer is managing his or her operation well, an assumption that is, unfortunately, probably overly generous in many cases. Nevertheless, perhaps the largest improvements that could be made in forage breeding overall would be the clear delineation of the key characteristics that make the ideal feedstuff for animals, and the development of robust, high-throughput assays, thereby clarifying breeding objectives and making selection possible (Kingston-Smith *et al.* 2013).

## Forage breeding successes

Throughout the 20<sup>th</sup> century, new forage cultivars have been developed by scientific breeding but also by simply releasing plants collected somewhere in the world, possibly from real-world forage systems. The cultivars released over the past century offer some combination of the three primary traits – better nutritive value, improved yield, and superior persistence – though capturing all three traits at optimal levels in a single package remains the holy grail of forage improvement.

Ecotypic selection continues to be a sound breeding strategy for many temperate forage species in many parts of the world. Numerous annual legume species have been trialed in Western Australia, and successful cultivar

releases directly from germplasm collections have occurred (Nichols *et al.* 2007). In North America, Joe Bouton and his colleagues released a highly successful white clover cultivar, ‘Durana’, derived directly from collections made in central Georgia (Bouton *et al.* 2005). Numerous other examples exist. For the more intensively selected species – such as alfalfa or perennial ryegrass, ecotypic selections are unlikely to become cultivars, and even for species such as white clover, most further advances will come from hybridization among germplasms and/or selection within desirable populations.

The simplest and probably still the most widely used breeding method is phenotypic recurrent selection of spaced plants grown to enable observation and recording of data on individuals. This method has obvious appeal – it is simple, it is easy, and it requires a minimum of infrastructure. It is also the method most different from that of the densely planted sward in which real-world forages are grown. Consequently, although high-heritability traits under additive genetic control are often improved quite well (the high levels of multiple disease and insect resistances in modern alfalfa cultivars are a well documented case), other traits, such as yield, are not so easily measured on individual plants, and not surprisingly, yield gains have been less than stellar in many forages (Brummer and Casler 2013).

The primary alternative to phenotypic recurrent selection is to implement some form of family selection, with half-sib families being most common. Judicious selection of family selection method can improve breeding effectiveness, even in the absence of other improvements (Casler and Brummer 2008; Resende *et al.* 2013; Vogel 2013). At least theoretically, genetic gain can be further improved by simply altering breeding methods to increase the selection intensity, improve parental control, or minimize environmental or genotype  $\times$  environment interaction.

The best example of temperate forage breeding success is the perennial ryegrass program at IBERS in Aberystwyth, Wales, UK, which has realized yield gains of 9% per decade, concurrent with improved quality while maintaining or improving persistence (Wilkins and Lovatt 2011). This is a stunning achievement, resulting from three key aspects of their program: (1) highly controlled, within-population improvement, leveraging both single plant and family evaluation, (2) evaluation of yield in sward plots under typical stand densities farmers would sow, and (3) a dedicated, long-term program that has enabled concentration of favorable alleles and the ability to capitalize on small incremental improvements across multiple cycles.

Synthetic cultivars are typically commercialized for most temperate forage species. This breeding method makes capturing non-additive genetic variance difficult, but not impossible (Tamaki *et al.* 2007). Heterotic effects for particular traits could result from hybrid production, but hybrid development is difficult with most forages – a lack of inbred lines and often high ploidy levels limit the implementation of canonical single-cross hybrids commonly developed in maize and many other crops. Yet methods to produce various types of hybrids have been proposed over the past 15 years (Arias Aguirre *et al.* 2012; Brummer

1999). Population hybrids (or “semi-hybrids” or “chance hybrids”) have been proposed numerous times, but the advantage of them is that they can be developed using existing technologies without relying on self-incompatibility and/or male sterility. While the heterotic boost obtained might not be as strong as a single-cross hybrid, nevertheless, the gain could still be significant (Brummer 1999). As characterization of self-incompatibility systems has improved (*e.g.*, Riday and Krohn 2010), methods to harness SI in hybrid cultivar development may be closer to realization.

## Phenotyping

Phenomics (Houle *et al.* 2010) is the latest rage in plant biology (following genomics, transcriptomics, proteomics, and metabolomics) – and a cynic might say that plant biology has finally caught up to plant breeding. Phenomics, as understood in the genomics community, describes the evaluation of trait phenotypes in high detail, under highly controlled conditions. Phenomics, as a plant breeder would use the term, describes evaluation of germplasm and breeding lines in the field under multiple natural environmental conditions in order to measure traits of ultimate importance to a farmer, such as dry matter yield, forage nutritive value, or plant persistence across years, thereby facilitating selection of superior plants.

Phenomics, in the first sense, will be used to specifically measure aspects of ultimate traits so that the underlying genetic basis of the trait can be determined. Typically, phenomics implies “high-throughput” evaluation – many plants being evaluated (possibly at many time points) in order to thoroughly characterize the reaction norm of a given genotype or set of genotypes under a range of possible environmental conditions. (One can define the “phenome” of a given plant genotype as the entirety of phenotypes that could be produced under any conceivable environment throughout developmental time.)

From a plant breeding perspective, the important aspect of phenomics is the development of screening methods to help improve an agronomically important trait. Secondly, if this screening method can also lead to the genes (or at least genetic markers) associated with the trait, so much the better. From this perspective, the practical interest in phenomics is not so much being able to characterize a whole range of phenotypes, but being able to define specific phenotypes particularly important to cultivar development, and standardizing a selection methodology that is high-throughput, accurate, and inexpensive.

Numerous “standard tests” have been developed in alfalfa for a suite of traits, including insect and disease resistances, morphological traits, and others (see [naaic.org](http://naaic.org)). For these tests, standard check cultivars were identified, and precise protocols developed to assess the characteristic. In some instances, these tests can serve as selection tests as well; others are simply evaluation methods, but the key characteristic of the tests is their well defined protocol to test the trait. Developing similarly well defined selection methods for various traits in the major species would undoubtedly be helpful. An example of a clear phenotyping strategy for a complex trait is that aimed at improving winter-hardiness in alfalfa by enhancing freezing tolerance

(Castonguay *et al.* 2009). The result of applying this highly controlled protocol to alfalfa populations has been the routine improvement in freezing tolerance, and more to the point, winter-hardiness. Probably the biggest deficiency in most forage breeding programs is the lack of high-throughput, highly accurate phenotype evaluations for major traits like yield, drought tolerance, and others. Pooling of resources across programs (and countries) could help bring about revolutions in phenotyping were relevant parties to put their heads together.

Other limitations that restrict genetic gain in forage breeding are related to specific issues of forage crops. Consider a typical maize or small grains program, with large numbers of yield plots on which other traits, such as grain composition, lodging, and so on can be measured. These plots are typically grown under commonly used production practices – that is, they are seeded at normal planting densities that farmers would use. In contrast, forage plots tend to be quite different from typical production methods – that is, evaluations of individual plants or even seeded rows do not reflect the typical sward conditions that a grazer would use. However, the reason these are used is twofold – first, producing sufficient quantities of seed for sward evaluations may be difficult, without sacrificing an additional year per selection cycle (which in itself will reduce genetic gain). Second, forage plot harvesting equipment has lagged behind advances made in the grain and oilseed crops. For instance, a major advance in those annual crop breeding programs was the introduction of dual plot harvesters, essentially doubling the amount of data that could be obtained for a given time frame.

For forages, harvest is a major undertaking, with multiple harvests per year and individual trials lasting several years. A dual plot harvester would greatly expedite data acquisition and enable larger numbers of plots to be handled. Third, even if yield harvests can be conducted, forage moisture and nutritive value cannot be easily obtained. New machines with on-the-fly NIRS measurements have been deployed (*e.g.*, by Haldrop), and this helps, provided the equations are suitable to the material being harvested. Moisture seems to be easily estimated in ryegrass trials with species composition also reasonable robust (R. Hayes and A. Lovatt, IBERS, pers. comm.) Nevertheless, the throughput on forage harvest is much lower than for grain harvest, and the weigh systems less precise and more affected by wind.

Remote sensing offers a potential to significantly improve aspects of forage breeding. For example, estimating biomass before and after grazing periods can provide an estimate of animal preference, and also an idea of yield. If this could be estimated remotely and non-destructively, considerable time would be saved (and in fact, data collected that often currently simply is not). The general topic of high-throughput, field-based, remote-sensed phenotyping is one of considerable interest for all crops (White *et al.* 2012), and explorations of the potential of various sensors to assess traits like yield, ground cover, species composition, and nutritive value are just beginning in forage crops (T. Butler and M. Newell, Noble Foundation, pers. comm.).

## Molecular markers

Easily observed morphological markers have long been used (or at least sought out) in breeding to enable indirect selection for an otherwise hard-to-select trait. Unfortunately, few morphological markers have been identified in most forage species, and those are typically detrimental to performance. Beginning in the 1970s, isozymes offered more plentiful and useful markers, but not until the 1980s, with the description of DNA fragment polymorphisms, did markers really show promise for genetic manipulation. By the early-mid 1990s, the major forage species had at least some DNA-based marker analyses underway. In 1993, two alfalfa genetic maps were published (Brummer *et al.* 1993).

Since these first maps back in 1993, DNA-marker based maps have become ubiquitous, developed for virtually all major temperate forage legumes and grasses, see, for example, recent maps in alfalfa (Li *et al.* 2011), ryegrass (King *et al.* 2013), and white clover (Griffiths *et al.* 2013). Genetic linkage maps are in-and-of themselves not particularly useful for plant breeding programs. Their value depends on the extent to which marker are associated with traits of interest. And here again, phenotyping comes into play; if a trait cannot be adequately phenotyped, then finding markers associated with the trait will not be successful. On the other hand, if phenotyping can be accomplished simply and easily, then the marker-trait association is of less consequence because the phenotype can be readily assessed. The middle ground, where phenotyping can be done accurately but the assay is either slow or laborious, is the sweet spot where marker-trait associations are highly useful. A clear example would be root traits (*e.g.*, Gregory *et al.* 2009))

One of the stumbling blocks to mapping, particularly of polyploids, was a dearth of markers, or at least, a limitation in the time and money to build saturated genetic maps. This limitation has been overcome by methods of genotyping-by-sequencing that can generate hundreds of thousands of SNP markers in a very short time. We have recently developed saturated alfalfa maps with several thousand markers in about a month, from start to finish, in both diploid and tetraploid populations (Li *et al.* unpublished). A similar approach was used to map fatty acids in ryegrass populations (Hegarty *et al.* 2013). Many additional projects are underway in the major forages.

Breeding programs offer a unique resource for identifying major genes (or markers for those genes) – divergently selected populations or populations selected for multiple cycles targeting one or few traits. We have recently used GBS to identify a likely region on chromosome 8 of alfalfa where allele frequencies shifted extensively during selection for whitefly resistance in alfalfa; phenotypic confirmation of this region as a resistance locus is underway (Monteros *et al.* 2013, IV Intl. Conf. on Forage Breeding, Melbourne). A similar strategy using possible candidate genes was recently used to identify rust resistance alleles in perennial ryegrass (Brazauskas *et al.* 2013).

As basic plant molecular biology continues to develop, identifying potentially useful “candidate” genes has gotten easier. Various genes have been identified for many major traits; whole biochemical pathways described in detail for

some traits, like lignin biosynthesis. Using these candidates enables breeders to determine if variation in gene sequences is related to traits – in the case of forage quality, sometime yes (Pembleton *et al.* 2013), sometimes no (Sakiroglu *et al.* 2012).

Using various mapping procedures, then, breeders have identified QTL for numerous traits in forage crops. But the great majority of these QTL have not been used in cultivar development programs to the author's knowledge. One exception is white clover seed yield (Barrett *et al.* 2009). Nevertheless, the use of markers for QTL manipulation has lagged considerably from the expectation those of had 20 years ago. The reason for this is probably related to several issues – the poor state of marker resources and maps, the relatively limited money available for marker research in forages, but perhaps most significantly, the difficulty in integrating markers for several QTL into a synthetic cultivar development program.

Today, with GBS, these issues are falling away. We now can easily cover the genome to focus not on individual QTL but on marker breeding values – selecting plants based solely on the aggregate marker values. While mining QTL from unadapted germplasm will undoubtedly be useful in the future, more potential seems to lie with genomic selection (Hayes *et al.* 2013; Li and Brummer 2012). By conducting marker-only selection using a model based on phenotypic data, multiple cycles of selection are possible during the several year cycle of typical field based breeding. Thus, the gain from the genomic selection program in aggregate across the cycles could easily outpace conventional selection. We have preliminary data suggesting model accuracies for yield selection across cycles of around 0.4 (Li *et al.* unpub.). If this holds up over several cycles, then we should be able to accelerate yield gain.

## Biotechnology

Much of the gene discovery research on crop plants, including forages, is geared to generating transgenes to provide new or expanded variation to cultivars. However, whether transgenes will ever become a significant aspect to cultivar development is questionable given current regulatory requirements in many parts of the world (Wang and Brummer 2012). Currently, RoundupReady alfalfa has cleared the legal hurdles and is being sold and grown in the US and elsewhere. Few additional products are in the pipeline, although modified cell wall composition and several others are contenders to be commercialized in the near future. Nevertheless, given the high cost of transgene development and deregulation, it's likely that only a few transgenes will ever be commercialized, and then, only in a select few species (alfalfa and perennial ryegrass being the most obvious choices).

That the power of transgenic modification won't be fully realized in forage breeding is unfortunate, but commercially viable traits based on transgene validation could be developed otherwise, *e.g.*, by Tilling or Ecotilling (Comai *et al.* 2004; Till *et al.* 2006). The search for induced or natural variants has been made easier by high throughput sequencing of amplicons from the candidate gene (Marroni *et al.* 2011). These strategies can be applied to heterozygous polyploids (Comai *et al.* 2004), which is

obviously of interest to many forage species.

## Proposals

Improving the genetic gain in forage crops for all traits will be important to deal with the major challenges facing the world. Drawing from the preceding discussion, let me summarize several main ways we can improve genetic gain – some at no added cost to the breeding program.

- (1) Improved plot design (see *e.g.*, Casler 2013). The good old randomized complete block design still remains a favorite of many breeders, despite ready availability of superior alternatives. Expanding the use of incomplete block designs (*e.g.*, alpha lattices), of spatial analysis (*e.g.*, nearest neighbor analysis), or unreplicated augmented designs could assist in generating better phenotypic data. In most cases, these types of designs will have minimal to no effect on field operations. Other experimental design changes could also be made to ensure that errors are appropriately small, facilitating discrimination among entries.
- (2) Know the traits. One may think that breeding programs have a clear idea of what traits to select, and in fact, a breeder may have an eye or a hunch that this or that trait is important. In some cases – such as a devastating disease – the critical trait is obvious. But in many cases, many traits seem important – disease resistances, drought tolerance, autumn yield, spring yield, cell wall composition, sugar content, and so on. Rarely are the economic values of traits teased apart, but if they are, then a clear idiosyncrasy can be quantified, as was done recently for perennial ryegrass (Shaloo *et al.* 2011). Collecting data is too time consuming for the wrong data to be collected.
- (3) Improved phenotyping. Knowing traits at least gives a target for the breeder, and his/her associates to aim at. But too often, the phenotypic data are not as robust as would be ideal, due to equipment limitations, time constraints, or poor technique. Some traits, like sugar content, are dependent on the time of day they are measured; thus, sampling needs to be done carefully. Other traits, such as biomass yield, can be hard to measure accurately when harvesting on windy days, for instance. And overall, speed of harvest limits the numbers of plots many breeders can evaluate. Consequently, if the traits can be measured more effectively, more rapidly, and more cheaply, then more plots can be planted, and (theoretically) more gain obtained. Application of NIRS and remote sensing equipment, as mentioned before, could help.
- (4) Improved breeding methodology. It's interesting that significant improvements can be made even before we get to breeding methodology itself! Here, Glenn Burton's words with respect to his restricted recurrent phenotypic selection scheme are valid for all breeders: *"The requirements for success are four: (1) A uniform screening procedure to effectively assess the yield potential of each phenotype; (2) An intermating procedure that will produce most of the possible hybrid combinations among the selected phenotypes; (3) A continuing effort to improve both procedures, and (4)*

Work, motivated by the needs of a rapidly expanding world population.” (Burton, 1982). The continuing effort to streamline, enhance, and improve all aspects of the breeding process was a hallmark of Dr. Burton’s highly successful program of selection. His work improving bahiagrass yield is somewhat deceptive in that spaced plants quickly expanded to form mini-swards, so in other species that have less potential to expand by rhizomes or stolons, developing methods to evaluate seeded swards would appear to be critical to continued improvement for yield and other traits. In these cases, family selection is probably essential, and some methods are better than others depending on the circumstances (Casler and Brummer 2008; Resende *et al.* 2013).

- (5) Application of genetic markers. And now we get to markers. Manipulating individual QTL is problematic in synthetic cultivars, not impossible, but difficult in terms of ensuring the desired allele is incorporated and brought to a reasonable frequency in the population. If the alleles are important enough, then the effort is worthwhile. However, the prospect of manipulating many QTL alleles for multiple traits quickly becomes problematic, and the methods to fully take advantage of the mapping results in a breeding program isn’t clear (Bernardo, 2001). Nevertheless, mining large effect QTL from unadapted germplasm, confirming the presence of resistances for multiple diseases or pests, and other uses will make knowledge of particular marker-trait associations useful, even if their use to improve quantitatively inherited traits in recurrent selection programs may not be. But more importantly, with the development of genome-wide markers, the prospects to use markers for genomic selection based on marker breeding values holds considerable appeal.
- (6) Introduction of new traits or expanded variation with transgenes and tilling. The opportunities here will be very specific to key target traits, but could make a significant impact, particularly if regulatory procedures change to make transgenic crops more successful.

## Summary

**A key point:** successful breeding programs require a field-based evaluation component. This is the fundamental requirement that cannot be superseded by any amount of biotechnology, genomics, or bioinformatics. Therefore, from a pragmatic standpoint – that of a seed company interested in generating income from a new cultivar – the value that any new technologies (including better forage harvesters!) bring to a cultivar need to bring returns *in excess* of the core field based program. New technologies may be able to change the way field programs are conducted, of course, but ultimately, the ability to produce seed, the greenhouses necessary for controlled pollinations, the disease and insect screening infrastructure, and the field-based planting, maintenance, and harvest machinery all still need to be on hand.

**The second key point:** Never before have we been in a position to bring so many disparate tools to bear on forage improvement. The potential to accelerate genetic gain for many traits, including complex traits like yield, seems

better than even just a few years ago. We are standing at the edge of great strides in forage improvement. Enjoy the ride!

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