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Pasture molecular genetic technologies - Pre-competitive facilitated adoption by pasture plant breeding companies

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Plain English Executive Summary

The gross economic value of the temperate grazing industries, based on perennial ryegrass pastures, has been estimated at c. $8 billion per annum in Australia. Increases in productivity realised by this industry have the potential to significantly increase the returns to the economy. An adequate rate of genetic gain to the pasture based industries is crucial to generate on-going competitive advantage and industry sustainability. Genetic improvement is designed to increase the profitability of the livestock enterprise through increasing productivity and quality of pasture plants, so that benefits are markedly higher than the costs of additional inputs. The historical rate of progress in the genetic improvement in pasture plant breeding is generally regarded as low, estimates of up to 7% per decade having been made for perennial grasses. Forage plant breeding companies must be provided with the means to deliver both novelty and genetic gain at increased rates.

Molecular marker technology identifies and exploits pre-existing genetic variation in populations to accelerate and re-design breeding programs and is widely used in a variety of agricultural programs, but the complexities of pasture plant breeding has limited its use to date. Practical approaches have been identified and developed to enable incorporation of the molecular breeding technologies within established breeding programs.

From the experimental work performed within the project, extensive analysis using detailed phenotypic and genotypic data has been performed on a clonal ryegrass plant nursery and associated sward trials. This has enabled a collection of novel breeding approaches to be tested and elite crosses to be performed, selecting for improvements in both yield and quality traits. These project activities have demonstrated methods of applying advanced phenotyping and genotyping methods in pasture plant breeding. The project has developed computational tools to assist in cross selection and new advanced phenotyping protocols that, critically, are cost effective for plant breeders, as well as novel cultivar sub-selection approaches that can expedite delivery of advanced products.

The capability for DNA based varietal discrimination and genetic integrity surveillance in pasture grasses has been developed and exemplified through construction of a detailed catalogue of relationships between ryegrass cultivars. This provides a degree of quality assurance and certification that can be deployed in the seed supply chain for ryegrass cultivars today. This outcome will benefit pasture breeding companies, through provision of definition and security of elite cultivars as well as describing the respective relationships between cultivars, which provides knowledge to select genuinely distinct germplasm for breeding programs. The DNA profiling technique will also benefit end-users in the pastoral production industries through increased confidence in dependability of the end-product.

Routine use of robust systems for quality assurance of novel endophyte status of ryegrass seed and tiller material has been implemented within a commercial breeding program. The application of endophyte QA/QC has directly identified levels of contamination in pre-commercial breeding lines for the utilisation partner. Toxic endophyte incursions can cause animal health and welfare issues as well as a loss in productivity. This outcome supports one of the largest suppliers of high-performance ryegrass genetics to the Australian dairy industry to deliver their product with increased confidence for the benefit of the end-user.

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1 Plain English program deliverables

As a direct result of project activities a number of key applications, tools, actions and outcomes have been delivered.

A large ryegrass clonal nursery has been established and all plants within the nursery have been measured for their performance for many key traits along with extensive DNA profiling. This has identified significant variation within current elite cultivars that can be further exploited. Several computational breeding tools have been developed that can assist in the selection and discrimination of these plants. The computation packages are enabled through processing data relating to phenotypic performance and DNA profile, to select for plant performance whilst retaining critical genetic diversity. As a result of the developed tools and activities, a large number of elite plant crosses have been performed and seed is available to further evaluation.

Commercial ryegrass breeding has, to date, not adopted advanced phenotyping procedures for aspects of herbage quality on an individual plant basis. This lack of adoption is due to the high cost and throughput limitations associated with these methods. Within this project, tools for low-cost high throughput wet chemistry based methods for carbohydrate and protein quantification have been developed. The developed protocols have achieved an 11-fold increase in throughput and a c. 74% reduction in price per sample. In addition, proof-of-concept experimentation has been performed on image-analysis methods to derive in-field estimates of ryegrass biomass through image-processing. Correlations of 0.77 to the actual realised plant biomass, measured by cutting and weighing the plant, have been achieved. These outputs represent a significant advance in the tools available to ryegrass breeding that can assist in increasing the traits selected.

An advanced cultivar catalogue for ryegrass has been developed for the Australian pasture industries. The catalogue is currently comprised of eight Italian ryegrass, one hybrid and nineteen perennial ryegrass cultivars (2 tetraploid and 17 diploid). All cultivars can be uniquely identified. Following the development of this initial catalogue, a more cost effective seed based method has been developed that delivers similar resolving power for a c. 80% price reduction. A second cultivar catalogue comprised of the same samples has been developed using this method. The application of this developed technology and data set has received significant interest from the utilisation partner and future use and uptake in commercial breeding programs is anticipated.

The implementation of a multiplexed QA/QC screening procedure for endophyte presence and integrity in ryegrass seed and tiller material has directly identified levels of contamination in pre-commercial breeding lines. The identification of these contaminations and low incidences of endophyte presence has enabled the utilisation partner, New Zealand Agriseeds, to directly address these issues to improve the quality of the seed that will be available for sale. In addition the detection and quantification of endophyte presence has been integrated into the ryegrass host DNA profiling techniques to deliver an integrated protocol. Routine integration of endophyte QA screening into the utilisation partners breeding practices is anticipated.

2 Project background

The gross economic value of the temperate grazing industries, predominantly based on pastures comprised of perennial ryegrass and white clover, has been estimated at c. $8 billion per annum in Australia, and due to the export-driven nature of the industry, increased pasture quality, persistence and productivity has the potential to significantly increase the returns to the economy.

An adequate rate of genetic gain is critical to the dairy, beef and sheep industries in order to generate on-going competitive advantage and industry sustainability. Genetic improvement is designed to increase the profitability of the livestock enterprise through increasing productivity and quality of pasture plants, so that benefits are markedly higher than the costs of additional inputs. A sound genetic improvement program provides the means to deliver this on-going genetic gain. It is essential that effective breeding programs, which are able to deliver genuine improvements in genetic gain and plant productivity are established, and that the respective industries can experience real benefit from the relevant research and investment. The historical rate of progress in the genetic improvement in pasture plant breeding is generally regarded as low, estimates of up to 7% per decade having been made for perennial grasses. The success of forage plant breeding companies is dependent on delivery of an ongoing pipeline of products, which can be differentiated and branded, which is an essential factor that must be considered in order to achieve uptake and delivery. However, in order to 'future-proof' the feed-base for the dairy and other livestock production industries, advances are required in improvement of the rate of genetic gain and incorporation of novel traits for improved adaptation in a changing environment. Consequently, the forage plant breeding companies must be provided with the means to deliver both novelty and genetic gain at increased rates. Consultation with the companies has confirmed these needs and a desire to embrace molecular breeding technologies, as has been the case for other crop plant genetic supply industries.

Molecular marker technology is the strand of biotechnology that does not require transgenesis, but instead identifies and exploits pre-existing genetic variation in natural populations in order to accelerate and re-design breeding programs. In previous capability building research, co-funded by GGDF, Dairy Australia (DA), MLA and the Victorian Department of Primary Industries (VDPI), comprehensive suites of molecular markers have been developed for perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.), two important grass and legume species for dairy and red meat pasture production in Victoria, and in other temperate regions of the world. The specific technology is single nucleotide polymorphisms (SNPs), which have been shown to be associated with genes for important agronomic traits such as herbage quality, disease resistance and tolerance to environmental stresses and are capable of efficient, high-throughput analysis exploiting the start-of-the-art infrastructure at DPI's Victorian AgriBiosciences Centre. A broadly applicable and world-leading breeders' 'tool-box' of applications for the present SNP technology has been designed, that can deliver dramatic improvements to pasture plant breeding to assist the dairy farming industry. In parallel, research performed by VDPI and RBG/NZA has generated equivalent priority technology for the fungal endophytes of perennial ryegrass and tall fescue (*Lolium arundinaceum* syn. *Festuca arundinacea* Schreb.), which are essential for pasture grass persistence in response to pest attack and environmental stresses in Australasian environments.

One of the major challenges is to adapt these new technologies and application to long-standing breeding process and commercial practices used by the seed industry, which are essential for delivery of novel pasture plant genetics to end-users for industry benefit. Practical approaches must be identified to enable incorporation of the molecular breeding technologies, in order to build confidence in the use of such novel solutions. The resultant knowledge and experience may then be applied in a pre-competitive manner to the improvement programs of a range of progressive forage seed companies.

To deliver advanced innovative genetic products of the feed-base to Australian pastoral agriculturalists, pre-commercial engagement and highly effective partnerships with pasture plant breeding companies, who consequently represent the primary users of the technology and consist of a small number of global players, must be established. This project aimed to support adoption of these new technologies by the commercial breeding programs of one of the largest suppliers of forage seed in the Victorian and broader Australian market: the Royal Barenbrug Group (RBG) and New Zealand Agriseeds [NZA] a wholly-owned subsidiary company. The proposal delivers on significant prior investment by industry (including support from the Geoffrey Gardiner Dairy Foundation [GGDF]) and utilises leverage provided by both the Dairy Futures Cooperative Research Centre (DF CRC) and the Meat and Livestock Australia (MLA) Donor Company (MDC) scheme to implement commercial-ready applications of both host grass and endophyte molecular breeding technologies for the benefit of the dairy and red meat industries.

There are currently no other nationally-based programs addressing pasture plant molecular breeding for the target species in this proposal. The project team are peer-recognised as part of the leading international group in forage molecular biology and genetics and have generated and tested the most advanced ‘tool-box’ for improvement of pasture grasses and endophytes. Pasture plant breeding companies such as RBG/NZA have identified VDPI as the ‘partner-of-choice’ for research and development joint ventures, as compared to other international agencies. The key point of differentiation in the proposed project and developed strategy, which differs from other international competitor research groups, is the seamless integration of the “tool-box’ of technologies into the company breeding programs without disruption of their established breeding practices.

3 Project objectives

The desired outcome that is addressed by this program is to increase the rate of genetic gain in pasture plant breeding in order to deliver novel cultivars, over reduced timelines, through the implementation of molecular genetic marker technology for the benefit of the Australian grazing industries. The specific project objective was to facilitate and assist the adoption of molecular genetic technologies in pasture plant breeding company programs, such that the companies actively use and partner in the development and direction of future applications. The technologies would specifically focus around the delivery of state-of-the art genomics and phenomics technologies (including comprehensive multiplexed panels of informative SNP markers. as well as advanced low-cost high-throughput phenotypic assays). The technologies must be developed and implemented to deliver informative data at acceptable costs and critically, without changing the structure of the established NZA breeding program.

To achieve these aims, three specific sub-components were developed within the scoped project. These were:

- Utilisation of useful phenotypic variation and corresponding genetic marker diversity for agronomic traits such as herbage quality, persistence and environmental stress tolerance in pasture grasses, leading to accelerated genetic gain and significant increases in yield, quality and persistence.

- Capability for varietal discrimination and genetic integrity surveillance in pasture grasses. This process will permit a degree of quality assurance and certification to be deployed in the supply mechanism for pasture seed. The outcome will benefit the pasture breeding companies, through provision of additional definition and security of elite cultivar or unique germplasm identification, but will also benefit end-users in the pastoral production industries (especially dairy farmers) through increased confidence in dependability of the end-product.

- Routine use of robust systems for quality assurance of novel endophyte inoculation in pasture grass breeding programs. Such systems will permit the identification and elimination of toxic endophyte incursions, for the benefit of the dairy and red meat industry through improvement of animal health and welfare.

To address these project subcomponents the following objectives were developed:

- Establishment and exploitation of a field-based plant clonal nursery with replication based on germplasm sourced from the NZA perennial ryegrass and Italian ryegrass breeding programs, allowing derivation of performance estimates based on both individual plant assessment and sward performance. In concert with standard performance metrics, the development and validation of low-cost high-throughput phenotypic assays using the clonal plant nursery will be performed.

- Genotypic analysis of individual plants to provide critical underpinning data for varietal discrimination and genetic integrity surveillance in ryegrasses, which has until now been based on logistically complex and costly procedures. Application of the genotypic data set to assist with selection of elite individuals and the implementation of accrued data in crossing decisions.

- Routine implementation of SSR-based genotyping technology that has been previously developed for grass fungal endophytes of the genus *Neotyphodium* for quality assurance and certification in the NZA perennial ryegrass breeding program, along with the development of integrated molecular marker screening tools to co-assess the plant and endophyte genotypes.

To deliver these objectives the following experimental activities were devised and performed:

- Establishment of a field-based plant clonal nursery to a combined total of 1920 plants, with genotypic data from each individual plant with a minimum of 384 SNP markers in a multiplexed single-tube format using the Illumina GoldenGate™ oligonucleotide ligation-amplification (OLA) system. This assay system is highly cost-effective and high-throughput in nature. The 384-plex SNP assay allows description of population structure (within and between varieties) and allows both assessments of genetic relatedness between individuals and essential underpinning data for varietal identification and discrimination, which has been specifically requested by the utilisation partners.
- In addition to the genotypic description of population structure described above, putative diagnostic marker assays were performed on the plants under investigation.
- A spaced plant field trial was established, with a total of 4 clonal ramets (sown as replicates in a block design) of each individual plant (producing a combined total of 7,680 plants). The trial was routinely measured for dry matter yield and herbage quality at vegetative growth stages in late winter and early spring, and reproductive growth stages during summer.
- Assessment was performed based on phenotypic analysis and genotypic data and plant selections were made for crossing. Crosses were directed to germplasm of potential commercial interest and to deliver experimental combinations capable of delivering maximum genetic gain, either through sub-selection or targeted introgression. Decisions on specific material to be crossed was made in conjunction with NZA.
- Assessment of derived F_1 progeny families was and will be conducted in a mini-sward evaluation trial site. Swards were established containing either exclusively full-sibs, or half-sib individuals.
- After the F_1 progeny trials have been established and fully assessed, additional F_1 individuals or derived-F_2 material may be selected and used as additional inputs to the clonal nursery. Genotypes of value for further assessment will also be available to enter into multi-location small plot trials to be managed by NZA.
- A low-resolution genetic diversity screen of a broader range of cultivar samples was performed, to inform a broader assessment of current elite ryegrass cultivars.
- Detection of low endophyte incidence and toxic endophyte incursions during varietal development in perennial ryegrass and tall fescue was performed.
- Quality assurance of endophyte-inoculated varieties to ensure persistence and productivity for the end-user and reduced animal health and welfare problems.
- Development of an integrated ryegrass and fungal endophyte-derived genetic marker screening tool for identification and characterisation of cultivar and toxin profiles.

The vision of success described in the project proposal was that following completion of the two-year program the undertaken activities would have achieved full-integration of the host grass and fungal endophyte-specific molecular genetic marker technologies into the NZA/RBG programs (as appropriate), and would have

demonstrated an effective match to company practices with clear demonstration of value in varietal development, such as:

- Identification of useful phenotypic variation and corresponding genetic marker diversity for agronomic traits such as herbage quality, persistence and environmental stress tolerance in pasture grasses.

- Completion of a commercial test for cultivar identification. This system will be used for quality assurance and will establish a new standard for certification of new cultivars. This will be an important step towards more transparent and customer-friendly marketing of cultivars (ensuring higher reliability that seed product performance will matches the supplier's specification).

- Routine use of robust systems for quality assurance of novel endophyte inoculation in pasture grass breeding programs. Such systems will permit the identification and elimination of toxic endophyte incursions, for the benefit of the dairy industry through improvement of animal health and welfare.

4 Delivery of scientific objectives

Clonal nursery

Trial Establishment and Phenotypic Assessment

Discussions over input material for content of the clonal nursery were held with the utilisation partner (New Zealand Agriseeds [NZA]), and a final list of elite perennial ryegrass and Italian ryegrass plants was established, 8 cultivars being identified as the base populations for each species. The list included current cultivars, along with advanced cultivar selections from the NZA breeding program. Seed was provided by NZA for trial establishment. This was matched with an additional 64 plants from a range of characterised non-adapted diverse germplasm, as well as specific genotypes with extensive root growth capability, that have been previously genotyped and phenotyped, to contrast with the elite commercial material.

The supplied seed was germinated at the premises of DPI-Hamilton under glasshouse conditions. A total of 1024 perennial ryegrass plants and 960 Italian ryegrass plants were selected. Plants were then raised in the glasshouse to allow vegetative propagation, and 6 clonal ramets for each plant were established. All plant identities were recorded in a proprietary laboratory-workflow database and were allocated bar-codes for sample tracking and tracing. Leaf samples for DNA extraction and single nucleotide polymorphism (SNP) genotyping harvested and transferred to DPI–Bundoora.

A total of 4 of the 6 clonal copies corresponding to each ryegrass genotype were established in a field trial, generating a cohort of 7,680 individual plants. Extensive phenotypic assessment was then performed on the field-based plants.

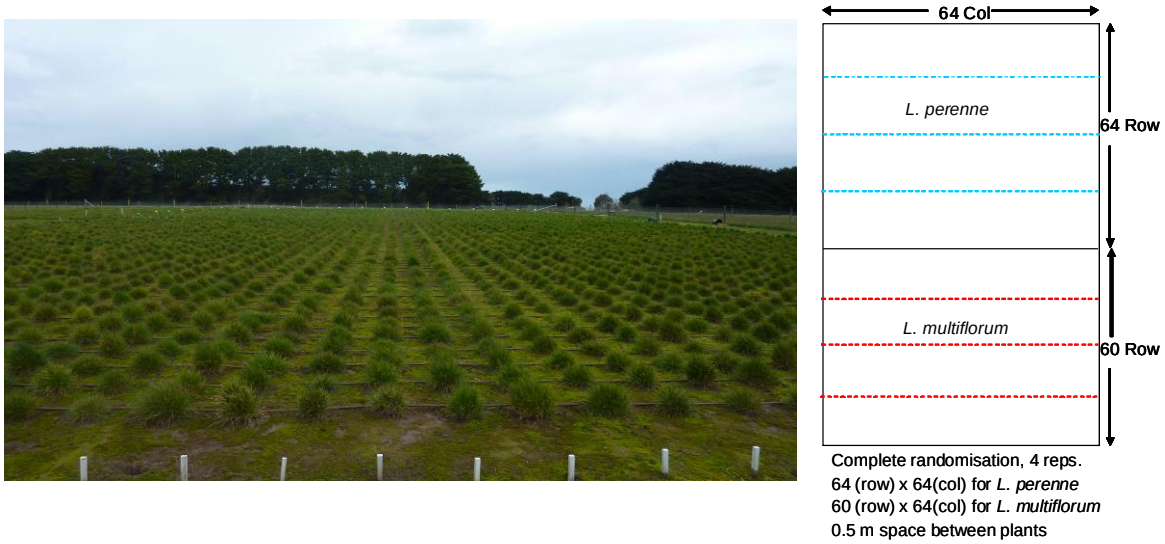


Figure 1. Image of the field-based clonal nursery, along with trial design and field lay-out.

Italian ryegrass spaced-plant trial

A total of 960 Italian ryegrass genotypes were assessed for herbage yield and nutritive values in a field-based nursery experiment located at Hamilton in south-western Victoria, Australia, in 2010-2011. The experimental design was a randomised complete block with four replicates, resulting in a total of 3840 plants in the trial. The field trial was established in the month of October 2010 (Figure 1). The monthly rainfall and mean soil temperature over the experimental period was recorded (Figure 2). Herbage yields (fresh weight:FW or dry weight:DW) were assessed from a total of 5 cuttings that were taken across all seasons and growth stages of the annual cycle. Heading dates were recorded during the 2010/2011 summer period, as well as morphological traits associated with flowering (length of the longest stem, length of the spike, flag leaf length and width of the longest stem, and spikelet number). Nutritive values including crude protein (CP), water-soluble carbohydrate (WSC), acid and neutral detergent fibre (ADF and NDF), and *in vivo* dry matter digestibility (IVVDMD) were assessed using near infra-red reflectance spectroscopy (NIRS) on vegetative samples harvested in May 2011.

Statistical analysis was performed using GenStat ver. 12. Correlation coefficients between the traits were calculated using the correlation command. Analysis of variance was computed using REML assuming block as fixed factor and genotype as random factor. Broad-sense heritability was then calculated as $H^2=\sigma_g^2/(\sigma_g^2+\sigma_\varepsilon^2)$, in which σ_g^2 is the genetic variance and σ_ε^2 is the residual variance. Repeatability was calculated as $R=\sigma_g^2/(\sigma_g^2+\sigma_\varepsilon^2/r)$, where r is the number of replicates.

Statistical analysis using REML revealed that block and variety exerted significant effects on yields and nutritive values. The effects of block and variety interactions were only significant ($p<0.05$) for FW-Jan and FW-Feb. The herbage yields from five cuttings were significantly correlated, however, the correlation coefficients varied from 0.05 to 0.72. The correlations between nutritive values CP, WSC, ADF, NDF and IVVDMD were highly significantly ($p<0.001$) either positively or negatively, with

the exception that there was not a significant correlation between ADF and CP (Table 1).

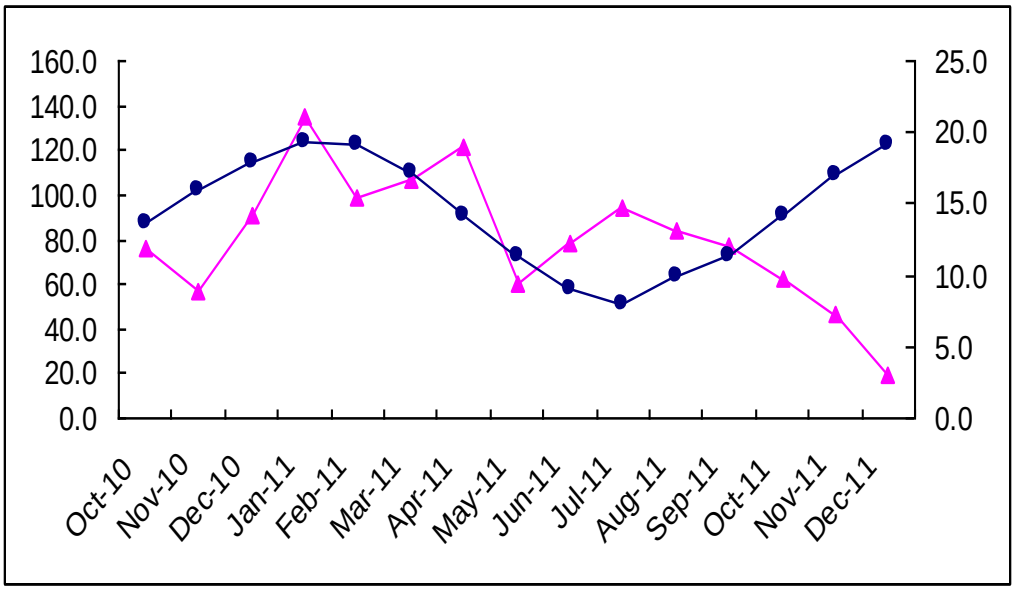


Figure 2. Monthly rainfall (mm, red line) and mean soil temperature (°C, blue line) at the DPI Hamilton field trial site during the experimental period.

Table 1. Correlation coefficients between yields and nutritive values of Italian ryegrass in a spaced field trial (* p<0.05, ** p<0.01, *** p<0.001, NS not significant).

	FW_Jan	FW_Feb	DW_Jun	DW_Sep	DW_Dec	CP	WSC	ADF	NDF
Fw_Feb	0.72***								
DW_Jun	0.30***	0.50***							
DW_Sep	0.05**	0.14***	0.54***						
DW_Dec	0.08***	0.07***	0.12***	0.34***					
CP	-0.10***	-0.10***	0.14***	0.37***	0.11***				
WSC	0.03^{NS}	0.04*	-0.10***	-0.32***	-0.15***	-0.77***			
ADF	0.10***	0.14***	0.08***	0.12***	0.04*	0.03^{NS}	-0.53***		
NDF	0.05**	0.01^{NS}	-0.05**	-0.05**	0.05**	-0.12***	-0.47***	0.75***	
IVVDMD	0.06**	0.14***	0.19***	0.13***	0.00^{NS}	0.14***	0.41***	-0.74***	-0.83***

Table 2. Variance, broad sense heritability, and repeatability of yields and nutritive values of Italian ryegrass in a spaced-plant field trial.

	σ^2_g±s.e	σ^2_ε±s.e	H^2	R
FW_Jan	1484.0±86.0	1542.0±41.0	0.49	0.79
FW_Feb	1728.4±89.2	794.4±21.3	0.69	0.90
DW_Jun	14.52±1.01	23.23±0.65	0.38	0.71
DW_Sep	21.16±2.14	83.06±2.34	0.20	0.50
DW_Dec	25.67±2.42	85.00±2.44	0.23	0.55
ADF	2.09±0.14	2.96±0.08	0.41	0.74
IVVDMD	3.36±0.22	4.62±0.13	0.42	0.74
NDF	5.26±0.31	4.81±0.14	0.52	0.81
CP	2.56±0.18	4.58±0.13	0.36	0.69
WSC	12.96±0.78	13.00±0.37	0.50	0.80

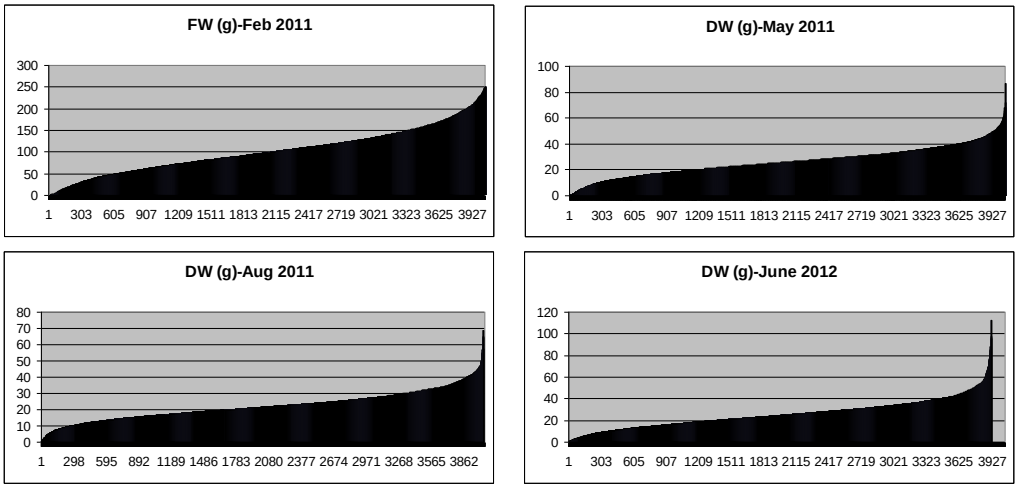
A broad range of phenotypic variation was observed, for instance, for vegetative yields and WSC content (Figure 3). Persistence varied between different varieties and genotypes, as well as seasonal growth (which are of interest in ryegrass breeding) in addition to the total annual herbage yield. There were significant genotypic variance components for yields and nutritive values. Moderate heritability estimates were obtained for nutritive values, as compared to low to high heritability estimates for yields from different cuttings (Table 2).

Perennial ryegrass spaced-plant trial

A spaced-plant trial including a total of 1024 perennial ryegrass genotypes was established in a randomized complete block design with 4 replicates, at the same location as the Italian ryegrass trial. The heading date of each plant was recorded during the 2011-2012 reproductive season. A total of 4 cuttings were performed as an assessment of herbage yield, and rank orderings of yield were obtained (Figure 3 and table 3). Plants were sampled twice within the duration of the project in order to assess WSC and total protein. Leaf tissue was harvested from each plant in August 2011 and June 2012, and the samples were analysed using a high-throughput protocol developed within the project (Figure 3) and described in the next section. In addition, the nutritive values of selected plants from cultivar Lp_D and breeding line

Lp_B populations were assessed using NIRS. During the summer of 2011 a natural infection of rust was present, and susceptible/resistant plants were visually assessed and scored in February 2011. In addition, a visual vigour score was conducted twice within the duration of the project these were performed in-between yield harvests.

(A)



(B)

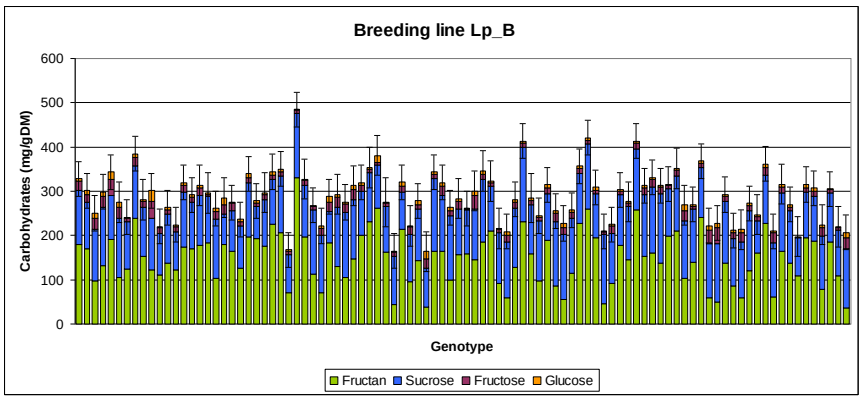


Figure 3. (A) Individual plant yields from five cuttings samples showing the range of variation. Each frame is sorted individually and no correlation of ordering is specifically preserved between frames. (B) Individual plant total water soluble carbohydrate content for breeding line Lp_B, as measured using an enzymatic procedure developed within the project. Each data point represents a plant genotype that is derived from the 4 clonal copies in the field, along with 3 technical replicates of the enzymatic procedure.

Table 3. Summary statistics describing the variances of yield and carbohydrate for the perennial ryegrass samples in the clonal nursery.

	Fw_Feb_2011_g	DW_May_2011_g	DW_August_2011_g	Fructan mg/g	Fructose mg/g	Glucose mg/g	Sucrose mg/g	Total_WSC mg/g
Number of values	4096	4096	4096	1022	1022	1022	1022	1022
Number of observations	4092	4044	3836	1022	1022	1022	1022	1022
Mean	104	26.2	22.31	152.9	16.52	8.208	131.7	307
Median	99.28	25.87	21.76	149.6	14.55	7.092	130.5	304
Minimum	0	0.04	0	8.854	1.004	0	58.33	139.2
Maximum	343.5	87.07	68.44	331.2	71.25	50.1	244.4	526.3
Range	343.5	87.03	68.44	322.4	70.24	50.1	186.1	387.1
Lower quartile	65.75	18.89	16.42	111.9	10.24	4.692	116.3	264.7
Upper quartile	136.4	32.89	27.66	188.4	20.62	10.67	145.5	342.9
Standard deviation	54.4	11.04	8.83	57.25	8.906	5.431	23.67	61.96
Variance	2959	121.9	77.98	3277	79.32	29.49	560.1	3839

Table 4. Correlation coefficients between yields and nutritive values of perennial ryegrass in a spaced field trial. P value test significance is shown in the lower portion of the table.

		FW_Feb_2011	DW_May_2011	DW_Aug_2011	DW_Jun_2012	Total_WSC_Au
	FW_Feb_2011					
	DW_May_2011	0.693	-			
	DW_Aug_2011	0.5419	0.6717	-		
	DW_Jun_2012	0.2542	0.35	0.2588	-	
	Total_WSC_Aug_2011	-0.0479	-0.0708	-0.0766	-0.1497	-
Two-sided test of correlations different from zero						
		FW_Feb_2011	DW_May_2011	DW_Aug_2011	DW_Jun_2012	Total_WSC_Au
	FW_Feb_2011	-				
	DW_May_2011	<0.001	-			
	DW_Aug_2011	<0.001	<0.001	-		
	DW_Jun_2012	<0.001	<0.001	<0.001	-	
	Total_WSC_Aug_2011	0.0051	<0.001	<0.001	<0.001	-

Statistical analysis was performed using GenStat ver. 12. Correlation coefficients between the traits were calculated using the correlation command. Analysis of variance was computed using REML, assuming block as fixed factor and genotype as random factor. The herbage yields from all cuttings were significantly correlated, but the correlation coefficients varied from 0.25 to 0.69, FW Feb 2011 compared to DW June 2012 and FW Feb 2012 compared to DW May 2011, respectively. A negative correlation between total WSC and yield was highly significantly ($p<0.001$) in all comparisons (Table 4).

Sward trial of perennial and Italian ryegrass

An early decision was made to establish field-based mini-swards relating to the plant cultivars that would be used within the project. For each of the base cultivars that entered into the clonal nursery, 8 replicate mini-swards were established, to a total of 128 plots. Each of the 8 perennial and 8 Italian ryegrass cultivars were sown in 1 x 1 m plots, to evaluate performance in conditions emulating standard field conditions at DPI-Hamilton (Figure 4). These mini-swards were extensively phenotyped, a total of 8 harvests being taken throughout the year. From each harvest, measurements were obtained for yield as well as a full suite of NIRS-calibrated measurements for herbage quality, including CP, WSC, fibre, as well as estimates of digestibility.

Figure 4. Mini-swards established in the field based on the cultivar material used as the input for the clonal nursery. Perennial ryegrass can be seen in the foreground, and Italian ryegrass in the background.

The experimental activities described have led to the development of a proposed breeding scheme that can capitalise on the success of current experiments and also allow maximum impact of advanced genomics and phenomics data measurement to

be realised (Figure 5). The proposed scheme includes a reduced generation interval between crossing steps, which will allow acceleration of genetic gain in breeding. Through implementation of a pedigree-based breeding system as described here, generation of estimated breeding values (EBVs) or genomics-derived estimated breeding values (GEBVs) can allow return of elite genotypes from the first round of crossing and selection directly into subsequent rounds of crossing cycles. The typical breeding cycle for a ryegrass cultivar is 12 years and the parents of subsequent cultivars often are derived from completed varieties. The rapid return of novel germplasm into a breeding nursery can dramatically increase the rate of genetic improvement in a breeding program.

The breeding scheme is designed to be cyclical with decisions on iterative crossing and selection to be informed by detailed performance and trait data as well as genotypic profiling. It is also proposed that cultivar breeding and release would be performed when sufficient genetic gain has been made to clearly distinguish a new 'pre-variety', followed by diversion of a sub-set of the selected genotypes into synthetic population development. Multiple polycross groups could be generated on the basis of predicted performance and genetic distance to produce restricted-base pre-varieties. Multi-site evaluation will still be required to measure phenotypic attributes such as yield, quality and persistence of these synthetics before commercial release.

This scheme is intended to deliver maximum genetic gain through rapid cycling of crossing activity based on predictions and performance data. The scheme also enhances the significance of individual genotypes during breeding practice, which has traditionally been negligible compared to populations as a whole.

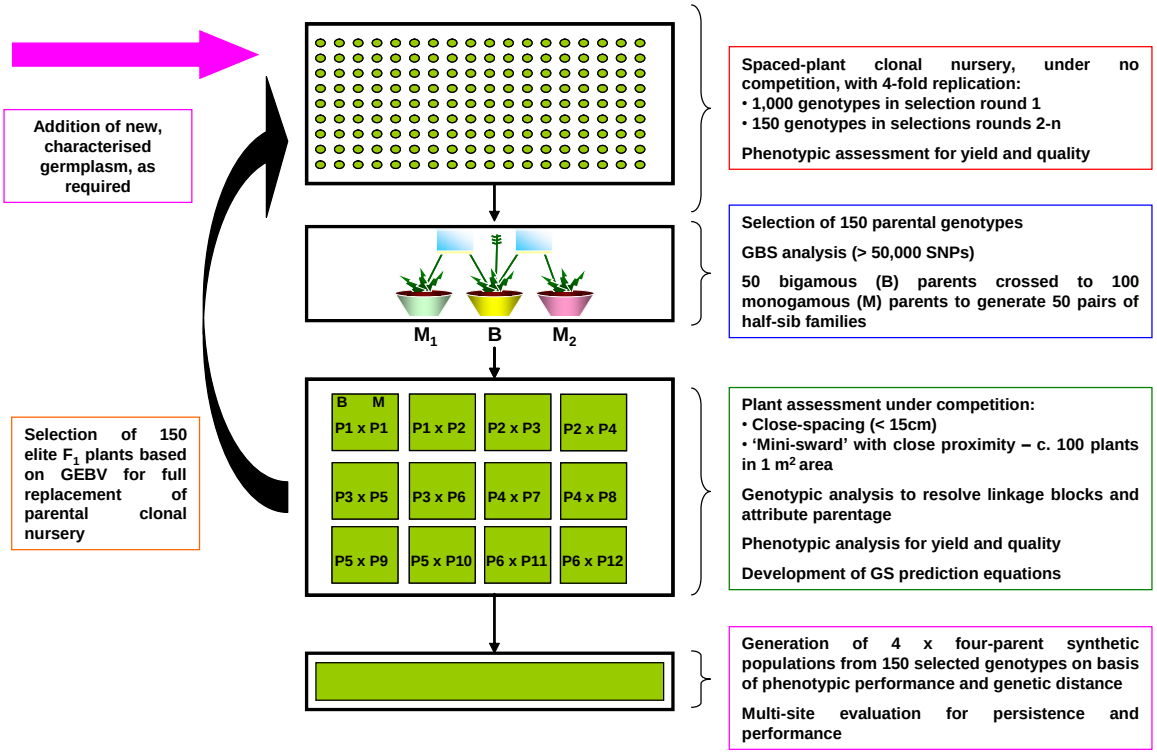


Figure 5. Potential breeding scheme designed and refined during the project that could implement genomic selection (GS) approaches into forage breeding. Design includes re-establishment and renewal of the clonal nursery along with introduction of novel germplasm and production of cultivar selections from specific crosses.

Development of novel phenotypic tools

High-throughput wet chemistry

A comprehensive review of the capability of forage quality assessment methods was undertaken. In order to advance the phenotypic assessment capability that was used in the project, efforts were made to develop a collection of high-throughput automated wet chemistry based protocols. Classical wet chemistry-based methods are neither high-throughput in nature nor cost-effective over large sample numbers, which has been a historical limitation in their uptake and application in breeding programs. A method of carbohydrate quantification that uses enzymatic and spectrophotometric methods was established. The method is amenable to automation and is also cost-effective. This method has the ability to process in excess of 400 samples per day and to return individual carbohydrate component levels. This represents a greater than 10-fold increase in sample-throughput when compared to traditional NIRS based protocols, with a dramatic reduction in cost per sample.

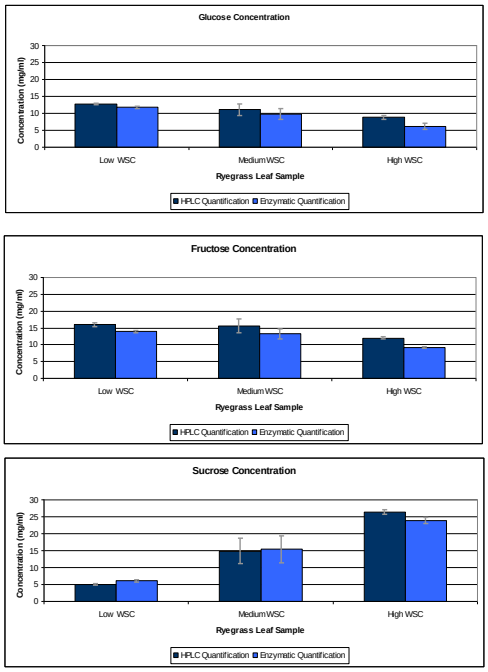
An estimate of project-based cost to perform NIRS based on processing internal to the organisation has been made at c. $11.40 per sample. This cost includes consumables, instrument depreciation and labour. Service delivery costs for NIRS based methods have also been obtained and are c. $60 per sample. An initial costing for internal project use of the enzymatic-based estimates of carbohydrate content has been made at c. $2.20 per sample, including consumables, instrument depreciation and labour.

All perennial ryegrass plants in the clonal nursery were harvested and assessed using the enzymatic protocol twice-over within the duration of the project. In parallel, all Italian ryegrass plants in the clonal nursery were harvested once and were analysed for quality measurements using NIRS-based methods (Figure 6).

Extension of the high-throughput automated protocols to additional traits was also performed. Quantification of total protein content was integrated in a ‘proof-of-concept’ experiment with the initial carbohydrate protocol. The combined protocol is complementary to the existing method for carbohydrate assessment, through use of the same extract as analysed for WSC content, which also contains extracted plant proteins. By integrating the extraction process with the WSC protocol, quantification of proteins can be performed in parallel. An initial costing for internal project use of the enzymatic reaction-based estimate of carbohydrate and the integrated estimate of true protein content has been made at c. $3.00 per sample (including consumables, instrument depreciation and labour), an additional 80 cents to integrate the true protein assessment.

Previous methods for WSC and protein quantification have been too slow and costly for effective commercial application in pasture breeding programs. However, with the development of these protocols breeders can access a rapid and cost-effective method for phenotypic assessment of large numbers of plants in their breeding programs, which will allow the development of cultivars with elite nutrient profiles (such as higher WSC, and lower protein content) that are better suited to the needs of grazing ruminants and can lead to lower input requirements and reductions in greenhouse gas emissions.

(A)



(B)

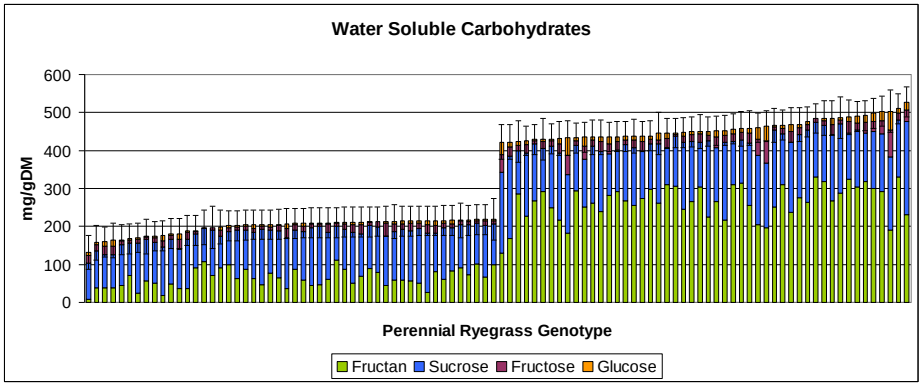


Figure 6. (A) Correlation of the developed enzymatic reaction-based assessment of carbohydrate content in ryegrass samples with HPLC quantification (B) Extreme tails of the distribution of the clonal nursery field trial, as assessed for carbohydrate content using the enzymatic reaction-based method.

Image analysis

In addition to the development of advanced wet chemistry based methods for calculating aspects of herbage quality, efforts have been made to develop image analysis protocols and capability to enable the estimation of herbage yield in a rapid and cost-effective manner. Images of a sub-set of plants from the field based clonal nursery were taken from above and the side and were assessed using specialist software packages, a total of 128 plants being assessed. The clonal nursery was planted based on a 50 cm spacing principal, each image being calibrated based on this plant spacing and a calibration of the number of pixels that equates to 50 cm being derived, for each image. The area covered by each plant was then calculated from each image, based upon the calibration (Figures 7 and 8). In addition to estimates of overall plant area, the side images were processed to derive estimates of plant morphology and measurements of plant height and width were obtained (Figure 8). Significant differences were identified in the morphology of different plant genotypes, with the largest value switching between width and height, as can be visualised in figure 8. It is anticipated that high-yielding plants with larger height than width, would lead to an overall higher yield under sward conditions, but plants greater spread would provide greater ground cover. Grazing styles of cattle vs sheep are quite different and do favour different growth habits, erect vs prostrate respectively, which can be selected and specifically bred for. In addition, genetic regulation of plant morphology erect vs prostrate is known to be highly heritable with estimates of broad sense heritability from the Italian ryegrass sub-set of the clonal nursery being 0.78, making this a highly tractable trait for future breeding targets.

Figure 7. Overhead image taken of four plants from the clonal nursery. Yellow lines surrounding each plant indicate the area measured from the image. Straight yellow lines indicate the distance between each plant based on a 50 cm spacing. Average values were taken from each image to derive an estimate of pixel value equal to a 50 cm distance.

Figure 8. Side-image of two plants from the clonal nursery. Red lines surrounding each plant indicate the area measured from the image. Straight yellow lines indicate the distance between each plant based on a 50 cm spacing to derive an estimate of pixel value equal to the 50 cm distance. Other yellow lines present within the measured plant area describe height and width of plants.

The images of the clonal nursery were obtained immediately following the perennial ryegrass harvest for herbage quality on 15th June 2012. The perennial ryegrass plants were subsequently harvested for biomass yield during the period from 18th - 21st June 2012. All plants were harvested and dry matter content was measured. From the image-derived estimates of area and other morphological measurements, correlations to the actual harvested biomass were made and correlation values up to 0.77 of image predicted mass to actual harvested biomass was obtained.

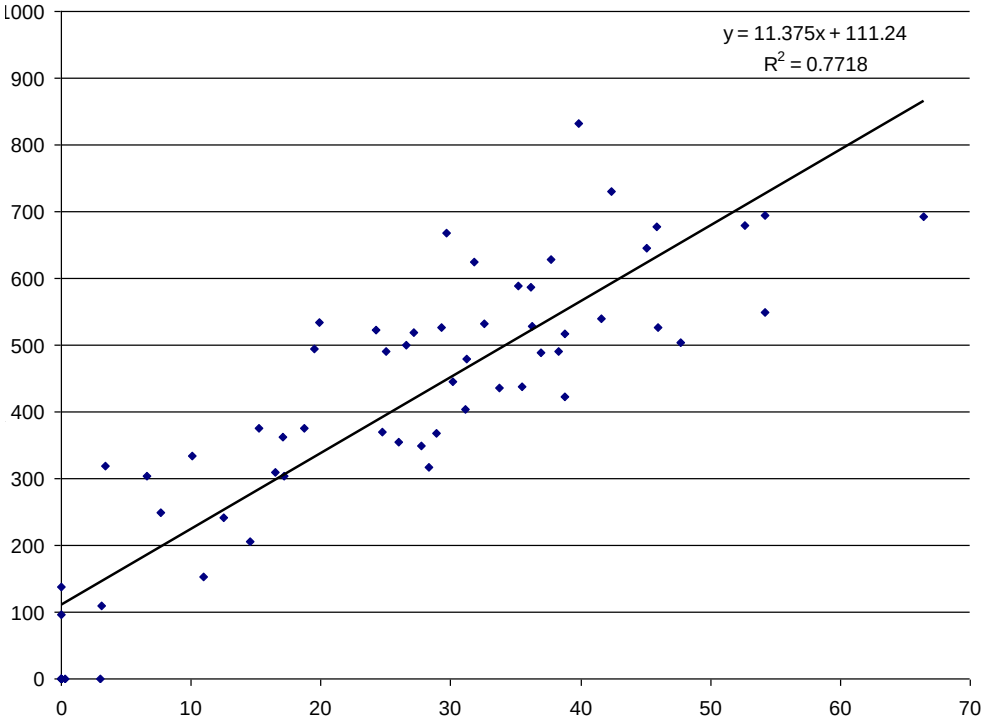


Figure 9. Scattergram displaying correlation of image-derived estimates of plant area plotted on the Y axis to realised plant dry matter biomass, plotted on the X axis.

In addition to the measurement of the established clonal nursery, initial analysis on the newly-established Italian ryegrass nursery was also performed. The second generation of the Italian ryegrass clonal nursery was established as spaced plant mini-swards, with closer spacing of 15 cm between plants, and each population represented as a 10 x 10 grid of plants. A single image of the population was taken and was automatically processed to derive estimates of total ground cover from the population expressed as a proportion of the image measured as grass following automatic image processing (Figure 10).

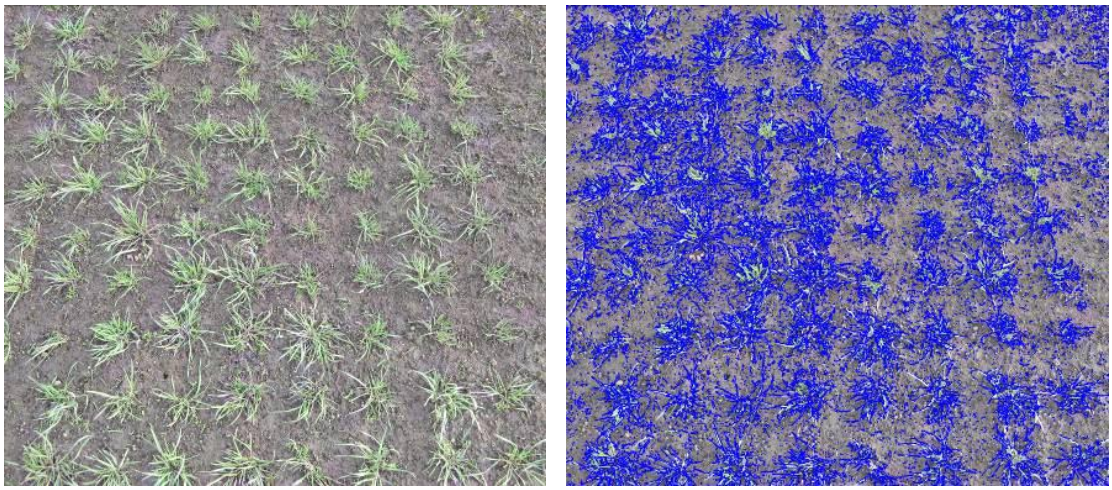


Figure 10. Overhead image obtained of a single population from the second generation of the Italian ryegrass clonal nursery. An unprocessed image is displayed on the left-hand side, with the processed image to the right. Blue colouration indicates the area measured following automatic image processing.

Genotypic assessment

All ryegrass plants (c. 2000 in total) constituting the clonal ryegrass plant nursery were genotyped with a collection of 384 pre-validated SNP markers, based on discovery activities from previously funded (DA/GGDF/MLA/VDPI-MPBCRC from 2003-2009) programs. These data provided an assessment of the genetic relatedness between each individual plant, as well as information on relatedness between each of the cultivars (Figure 11). The two species of ryegrass that constituted the clonal nursery, formed two separate clusters distributed along the x axis of the PCA plot, with greater overall variability being exhibited within the perennial ryegrass samples

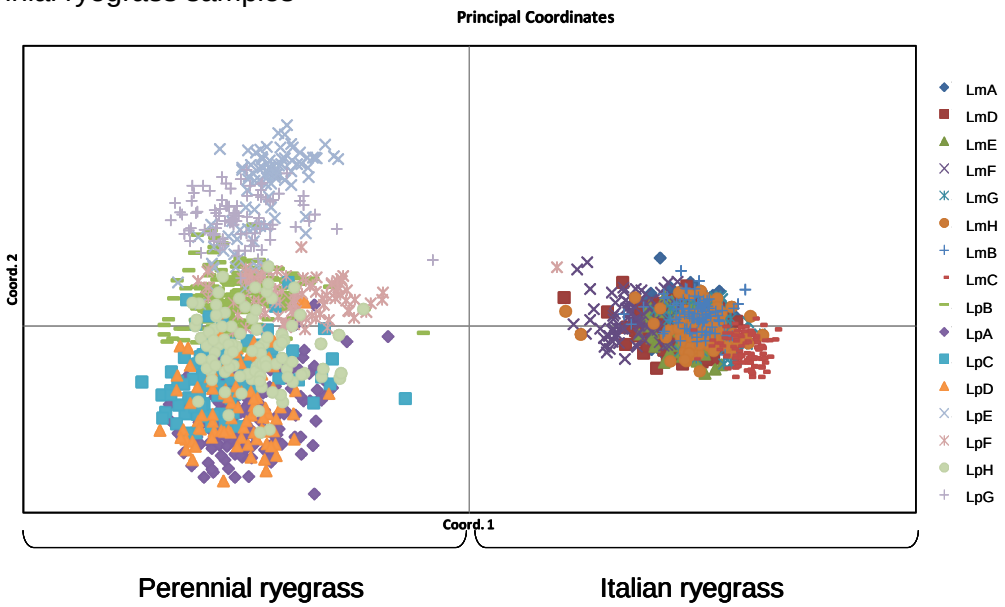


Figure 11. PCA plot of all samples from the clonal nursery following assessment with a pre-formatted collection of 384 SNP markers.

Detailed resolution of the relationships between cultivars and samples is difficult to obtain from the PCA plot when both species are represented, but significant structure can be identified when samples from a sole species are examined. The Italian ryegrass samples tend to cluster by cultivar, with some cultivars showing distinct overlap (Figure 12).

When considering samples from each cultivar as a population, a more simplistic view of relationships between cultivars can be established (Figure 13). The generated dendogram permits higher resolution between the cultivars that showed a large degree of overlap in the PCA analysis.

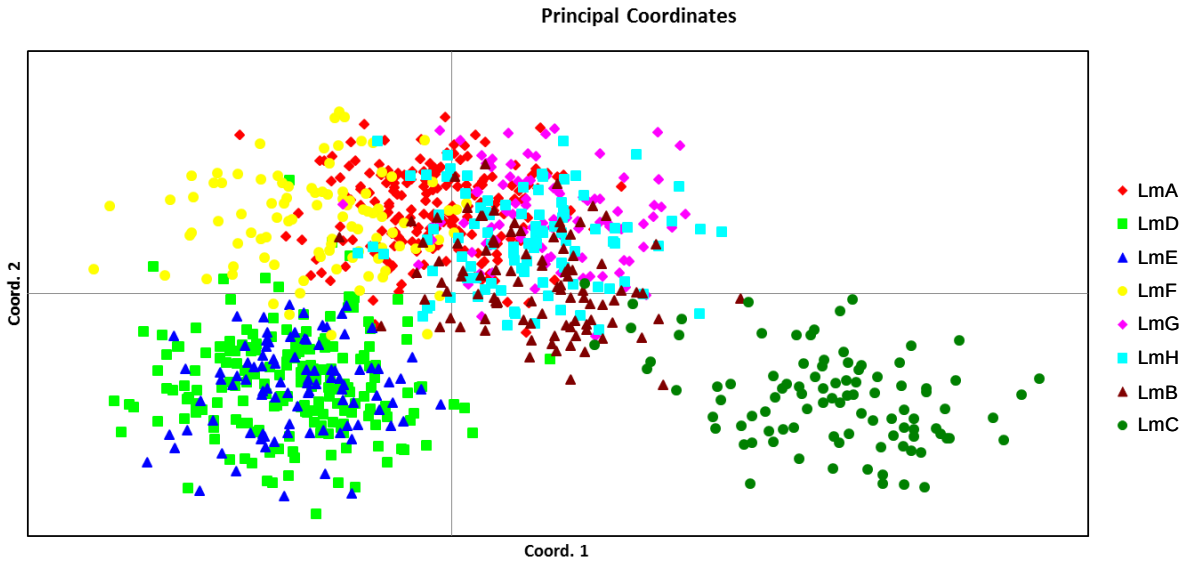


Figure 12. PCA plot for all Italian ryegrass samples from the clonal nursery assessed with a pre-formatted collection of 384 SNP markers.

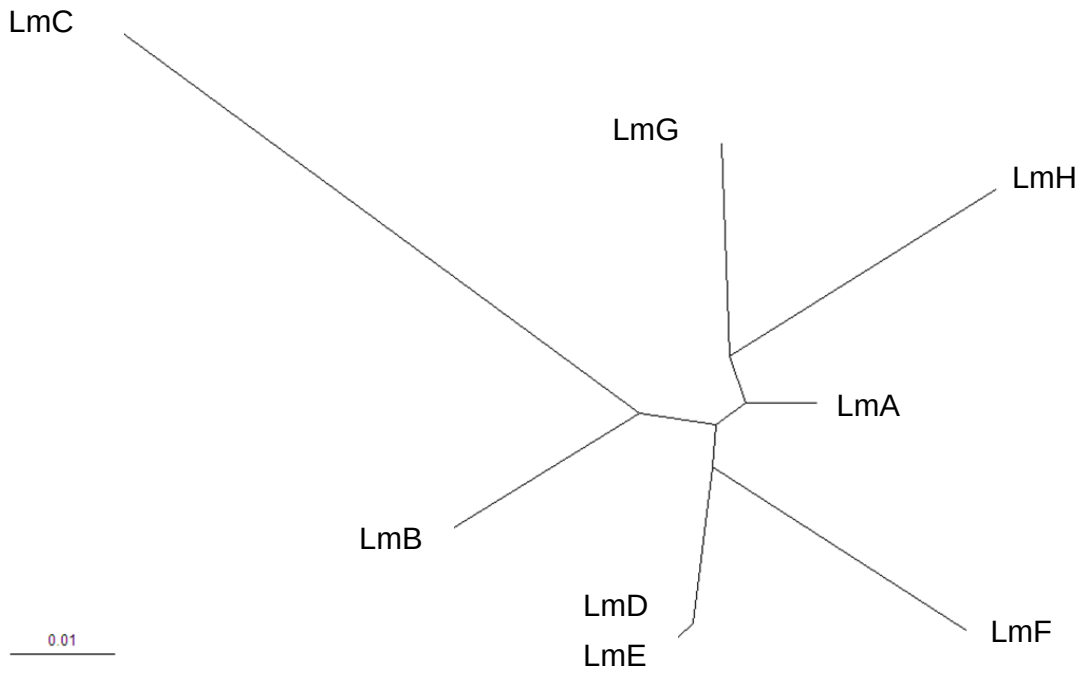


Figure 13. Neighbour joining dendogram of the relationships between Italian ryegrass cultivars defined as populations represented within the clonal nursery, when assessed with a pre-formatted collection of 384 SNP markers.

The perennial ryegrass samples when analysed using PCA, also cluster by cultivar, again revealing significant overlap between some samples (Figure 14). Cultivar LpE is seen as the most genetically distinct cultivar with substantial intrapopulation diversity.

Taking samples from each cultivar as a population, a more simplistic view of the relationships between the cultivars can be established (Figure 15). The dendogram permits higher resolution between the cultivars that showed a large degree of overlap in the PCA analysis. The cultivars LpA and LpC can be seen as most closely related, while the cultivar LpE is the most divergent population within the analysis.

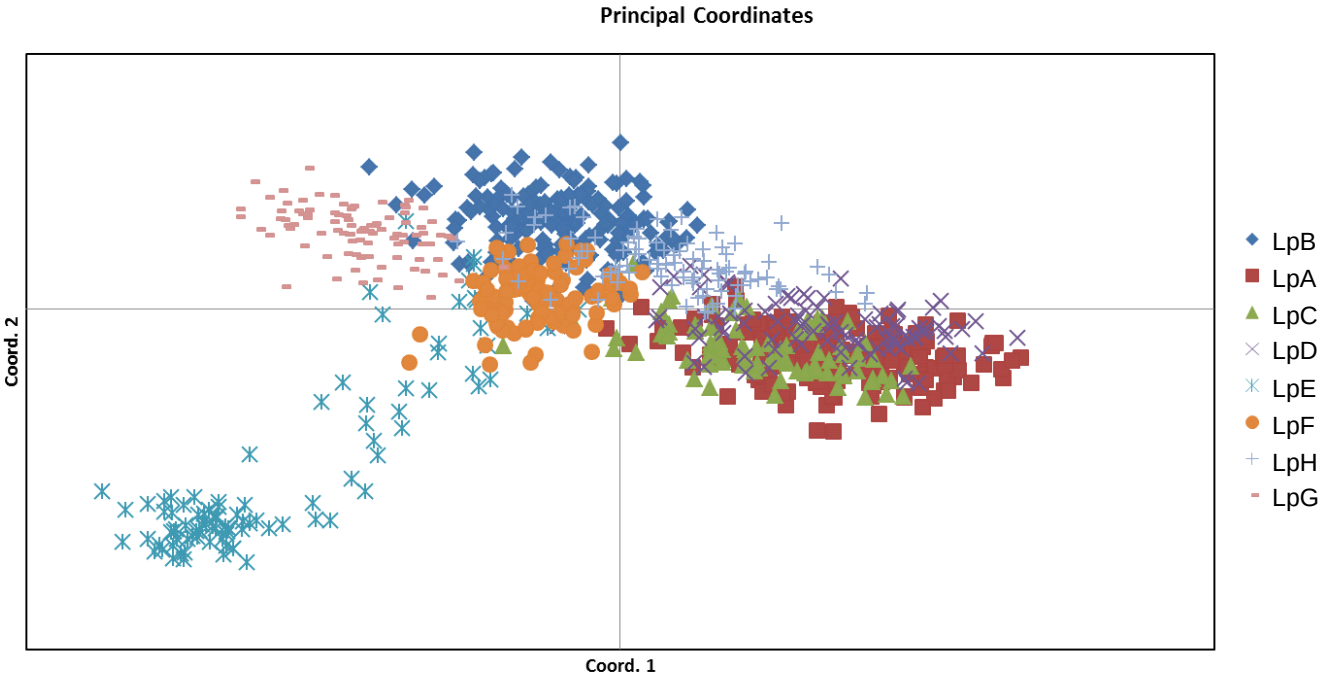


Figure 14. PCA plot of all perennial ryegrass samples from the clonal nursery, when assessed with a preformatted collection of 384 SNP markers.

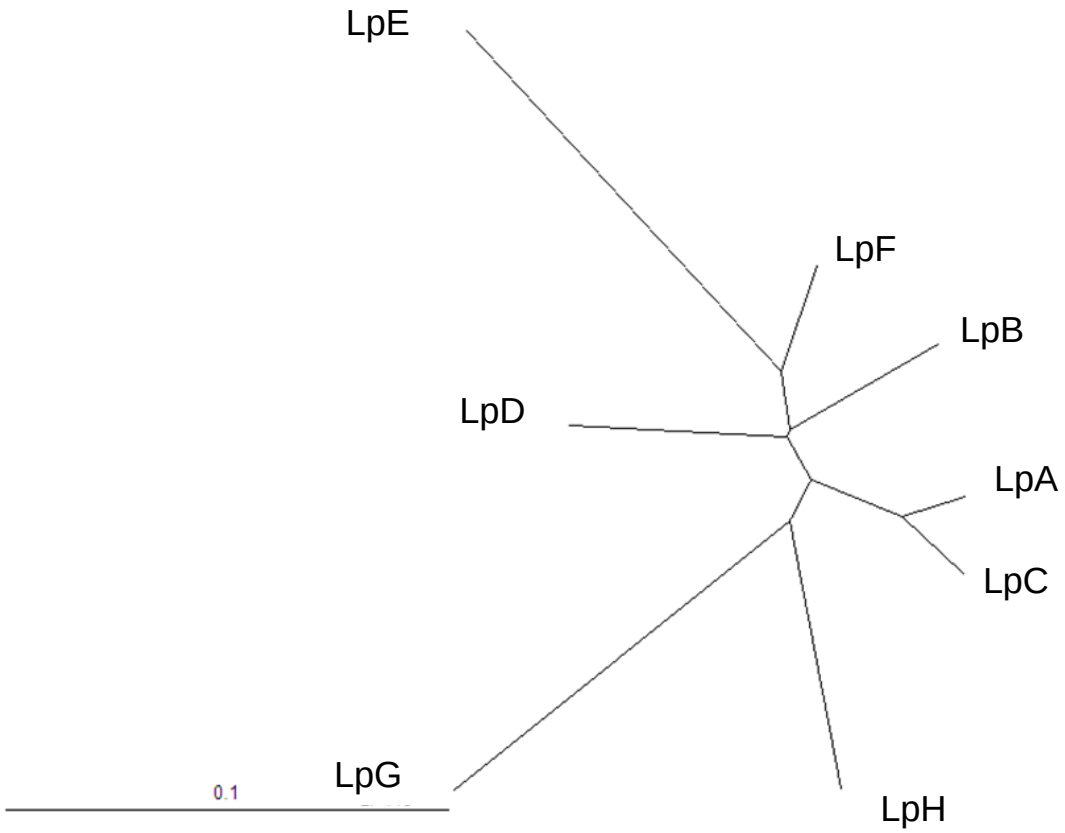


Figure 15. Neighbour joining dendogram of the relationship between the perennial ryegrass cultivars defined as populations represented within the clonal nursery when assessed with a pre-formatted collection of 384 SNP markers

All c. 2000 plants were also genotyped with a collection of 7 pre-validated SNP markers located in candidate genes for cell wall biosynthesis and oligosaccharide metabolism. The SNPs under investigation are derived from a previously performed association mapping experiment that had been conducted from research previously funded by DA/GGDF/MLA/VDPI-MPBCRC. The former experiment, which analysed a diverse metapopulation of perennial ryegrass plants (220 genotypes) was established with replication as a spaced plant field trial at DPI-Hamilton. The plants were genotyped with a larger cohort of SNPs (c. 100) from candidate genes involved in pathways of cell wall biosynthesis and oligosaccharide metabolism. Herbage samples were harvested at both vegetative and reproductive stages and were measured for a range of herbage quality traits using NIRS. The seven SNPs that were taken forward for genotyping in the current project were selected based on initial associations that had been identified in the data sets.

Table 5. Diagnostic SNP markers identified from previous related project based activities genotyped across all plants present in the clonal nursery.

All Plants	LpCAD2-4976f	LpCWinvAU136	LpFT3f336f	CcoaOMT-100	CcoaOMT-157	SPSf-160	SPSf-256
AA	461	25	616	1316	1593	392	1133
AB	488	343	340	393	220	627	563
BB	983	1552	604	215	100	853	160
--	52	64	424	60	71	112	128
% missing	2.62	3.23	21.37	3.02	3.58	5.65	6.45
All Italian							
AA	17	16	182	481	709	15	738
AB	121	172	70	255	147	204	149
BB	808	729	357	189	60	685	10
--	14	43	351	35	44	56	63
% missing	1.46	4.48	36.56	3.65	4.58	5.83	6.56
All Perrenial							
AA	444	9	434	835	884	377	395
AB	367	171	270	138	73	423	414
BB	175	823	247	26	40	168	150
--	38	21	73	25	27	56	65
% missing	3.71	2.05	7.13	2.44	2.64	5.47	6.35

Computational tools and crossing selection

Data handling – Computational cross and parent selection

With the advent of next-generation genotyping platforms and highly multiplexed SNP assays, the ability to rapidly and cost-effectively genotype plants in commercial breeding nurseries has become realistic and practical. This capacity not only establishes a pathway to genomic-assisted breeding of crops and forages, but also provides a direct application in current breeding program schemes. Maintenance of genetic diversity in breeding nurseries is fundamental to the ability to make continual genetic gain in breeding lines. Loss of rare beneficial alleles can be common when heavy phenotypic selection is applied, and this represents a potential erosion of future genetic gain. The ability to perform population identification and seed purity analysis from genotypic data also provides benefits to breeders. In order to fully realise the value of genomic technologies in plant breeding, effective computational tools are required for maximum exploitation, which is an area that is typically ignored in developing modern genomic-assisted breeding schemes.

Selection whilst Conserving Diversity (SeConD)

SeConD was written as a software package in the programming language R. SeConD automatically selects a subset of phenotypically elite individuals from breeding nurseries to take forward for establishment of subsequent breeding nurseries, but also ensures that all allelic variants present in the current nursery are conserved in the next. SeConD was evaluated on the Italian ryegrass component plants in the current clonal nursery, based on genotype data from 384 SNPs. A 15% selection pressure was applied, and SeConD efficiently selected 150 individuals that conserved all the alleles present in the current population while also permitting selection for elite phenotypes (Figure 16). Compared to selection solely on phenotype, the sub-set derived from use of SeConD was only 2% lower yielding than the top 150 yielding genotypes (Figure17). If selection was based solely on phenotype, 7% of the alleles present in the previous population would have been eliminated.

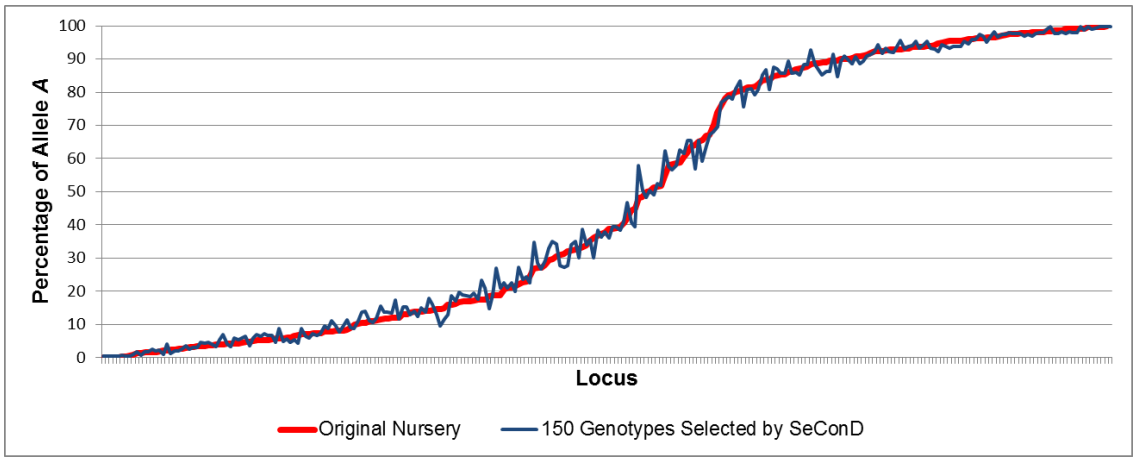


Figure 16. Effective conservation of allele ratios observed in the original clonal nursery population of Italian ryegrass plants by the sub-set of 150 genotypes selected by SeConD.

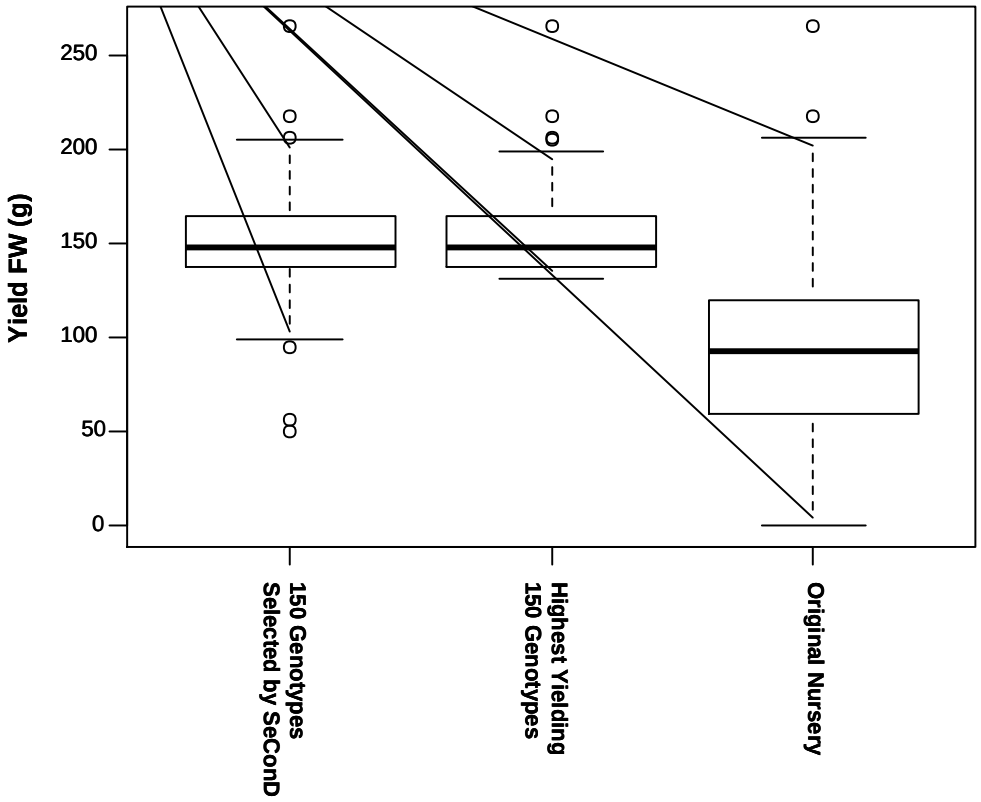


Figure 17. Fresh weight yield distribution of the 150 genotypes selected by SeConD, as compared to the 150 highest yielding genotypes and the original nursery.

Statistical Analysis of Mixed Ploidy Populations (StAMPP)

Currently there are no publicly available software packages for the analysis of population structure and differentiation with confidence intervals and p-values in mixed ploidy populations. StAMPP was written (in the programming language R) to enable calculation of Nei's genetic distance (Nei 1972) between individuals and populations, pair-wise F_{st} values (Weir and Cockerham 1984) with confidence intervals and p-values between populations, and the genomic relationship between individuals from mixed ploidy populations with SNP genotype-derived datasets. To validate F_{st} values generated by StAMPP, genotype data from 16 diploid and 2 tetraploid perennial ryegrass varieties and 2 hybrid ryegrass varieties were analysed for population differentiation in StAMPP and SPAGeDi (Hardy and Vekemans 2002). SPAGeDi is a widely used software package that calculates F_{st} values for population differentiation, but does not generate confidence intervals and p-values for multiple pair-wise comparisons. Comparison between the F_{st} values (Table 6) and Nei's genetic distance as reported by StAMPP and SPAGeDi revealed a high level of similarity and validated StAMPP as an accurate tool for mixed ploidy population differentiation. Small differences in the values generated between the two packages are explicable in terms of small differences in rounding numbers during calculations. Genomic relationship values between individuals were calculated following Yang *et al* (2010) and outputted as a genomic relationship matrix, which can then be inverted

and used to increase the power of association studies and genomic selection, and for prediction of breeding values.

Table 6. Comparison between reported pair-wise F_{st} values derived from StAMPP and SPAGeDi. The lower half of the table shows the StAMPP-derived values, while the upper half shows the SPAGeDi-derived values.

	Lp1	Lp2	Lp3	Lp4	Lp5	Lp6	Lp7	Lp8	Lp9	Lp10	Lp11	Lp12	Lp13	Lp14	Lp15	Lp16	Lp17	Lp18	Lb1	Lb2
Lp1		0.108	0.096	0.089	0.130	0.102	0.094	0.124	0.198	0.156	0.121	0.104	0.082	0.157	0.097	0.149	0.157	0.089	0.262	0.095
Lp2	0.108		0.068	0.060	0.105	0.097	0.091	0.090	0.140	0.134	0.097	0.097	0.096	0.180	0.088	0.112	0.135	0.153	0.268	0.063
Lp3	0.099	0.065		0.038	0.112	0.111	0.108	0.137	0.192	0.163	0.114	0.110	0.093	0.188	0.086	0.152	0.155	0.152	0.254	0.066
Lp4	0.089	0.060	0.036		0.120	0.106	0.090	0.139	0.201	0.167	0.115	0.104	0.090	0.188	0.069	0.151	0.159	0.155	0.297	0.060
Lp5	0.130	0.104	0.112	0.120		0.105	0.098	0.107	0.203	0.157	0.140	0.142	0.124	0.214	0.136	0.151	0.174	0.167	0.310	0.100
Lp6	0.102	0.097	0.109	0.106	0.105		0.047	0.074	0.144	0.090	0.093	0.104	0.108	0.184	0.117	0.119	0.129	0.108	0.278	0.079
Lp7	0.094	0.090	0.107	0.090	0.098	0.047		0.081	0.140	0.097	0.091	0.099	0.097	0.183	0.110	0.128	0.114	0.111	0.286	0.071
Lp8	0.124	0.090	0.135	0.139	0.107	0.074	0.081		0.113	0.104	0.109	0.120	0.122	0.202	0.143	0.095	0.129	0.151	0.291	0.114
Lp9	0.200	0.141	0.196	0.203	0.201	0.145	0.139	0.114		0.107	0.139	0.154	0.189	0.260	0.188	0.154	0.182	0.196	0.326	0.153
Lp10	0.156	0.133	0.163	0.167	0.157	0.090	0.097	0.104	0.109		0.112	0.136	0.151	0.230	0.172	0.149	0.142	0.174	0.316	0.137
Lp11	0.121	0.097	0.116	0.115	0.140	0.093	0.091	0.109	0.137	0.112		0.032	0.109	0.180	0.107	0.115	0.125	0.145	0.282	0.097
Lp12	0.103	0.096	0.106	0.103	0.143	0.103	0.100	0.119	0.155	0.137	0.031		0.057	0.100	0.064	0.094	0.121	0.145	0.259	0.094
Lp13	0.083	0.094	0.089	0.090	0.122	0.109	0.098	0.122	0.183	0.150	0.109	0.059		0.051	0.061	0.098	0.133	0.155	0.231	0.091
Lp14	0.157	0.179	0.190	0.188	0.214	0.184	0.183	0.202	0.262	0.230	0.179	0.100	0.052		0.102	0.138	0.203	0.240	0.332	0.181
Lp15	0.096	0.087	0.083	0.068	0.137	0.115	0.109	0.142	0.189	0.171	0.106	0.063	0.059	0.103		0.123	0.151	0.162	0.282	0.080
Lp16	0.150	0.112	0.151	0.152	0.149	0.120	0.127	0.096	0.151	0.149	0.116	0.094	0.099	0.138	0.123		0.150	0.186	0.296	0.128
Lp17	0.157	0.135	0.157	0.159	0.173	0.129	0.114	0.129	0.180	0.142	0.125	0.122	0.137	0.203	0.152	0.149		0.187	0.338	0.152
Lp18	0.089	0.153	0.154	0.155	0.167	0.108	0.111	0.151	0.194	0.174	0.145	0.146	0.154	0.240	0.163	0.185	0.187		0.281	0.123
Lb1	0.262	0.267	0.261	0.297	0.310	0.278	0.286	0.291	0.323	0.316	0.282	0.260	0.240	0.332	0.287	0.294	0.338	0.281		0.221
Lb2	0.095	0.062	0.064	0.060	0.100	0.079	0.071	0.114	0.155	0.137	0.097	0.092	0.090	0.181	0.081	0.130	0.152	0.123	0.221	

Crossing and plant establishment

Italian ryegrass

Application of the software tool SeConD was performed to permit the selection of 150 plants from the initial set of 960 Italian ryegrass plants within the clonal nursery. The selected plants were identified as accurately representing the breadth of genetic diversity that was identified in the initial population, while also representing a collection of high-yielding individuals to deliver an overall increase in biomass. This criteria was assessed in two ways: the presence of individual plants from all of the initial cultivars in the selection, as well as the presence of all of the genetic variation identified in the population as assessed by the collection of SNP molecular markers. This specifically ensures that all of the variable molecular genetic marker alleles that were identified have been represented in the selection, potentially at varying rates, but with no erosion of genetic variability. These plants were cloned from glasshouse-located clonal replicates and vernalised for 6 weeks. A total of 100 pair-crosses were conducted under glasshouse conditions. To ensure provision of sufficient seed yield for subsequent progeny testing, the same pair crosses were also performed under field conditions, and some crosses were also performed in a hydroponic culture system (Figure 18).

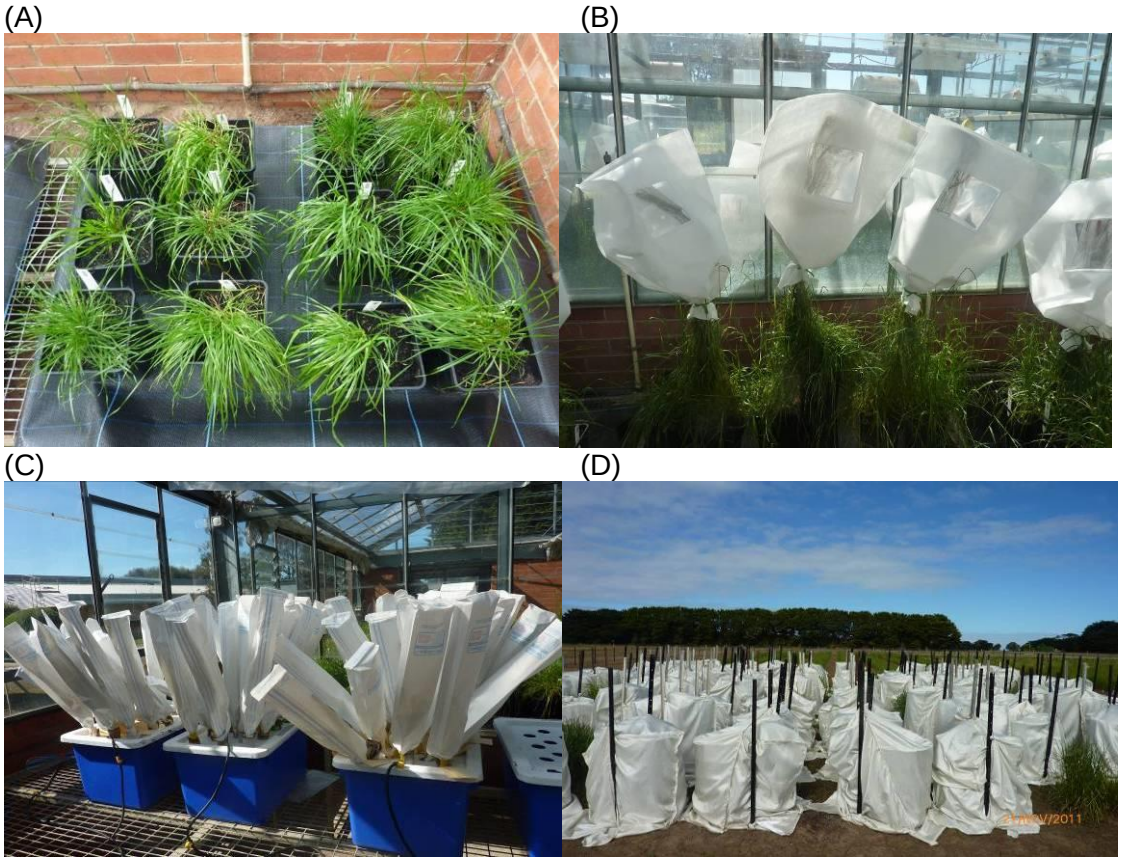


Figure 18. Crosses performed with selected Italian ryegrass plants. (A) Plants arranged for pair-crossing in glasshouse space after vernalisation for 6 weeks. (B) Crossing bags used for inflorescence isolation under glasshouse conditions. (C) Hydroponics-based crossing and (D) field-based crossing.

A total of 100 pair crosses were performed using multiple clones and significant seed yield (over 400 seed per cross) was obtained for 94 of the crosses. The resulting seed was cleaned and germinated, to establish 100 plants per cross, to a total of 9,400 plants. These plants were then transferred to the field once an appropriate growth stage was attained. The field trial was established as a spaced plant mini-sward, with individual plots for each of the crosses along with the original cultivars as a comparison. Between plots or populations, 1 m gaps were preserved. The population plot was established with 15 cm spacing between plants. This register was chosen in order to enable individual plants to be identified, but also provide competition effects between plants.

An initial test of F_1 family performance was planned as a field-based plot experiment to include the 94 F_1 families that generated over 400 seeds, along with the 8 original cultivars. A total of 110 plots were included in the design, 2 from each of the original cultivars being included and one each from the generated F_1 families. A total of 200 seeds from each of the 94 F_1 families derived from pair-crosses of Italian ryegrass and 300 seeds from the 8 original parental cultivars were geminated in seedling trays (100 cells per tray) in the glasshouse. The average germination rate was 93%, with a range from 67% to 100%. Seedling vigour was assessed based on tiller number and leaf number of each seedling. In addition all seedlings biomass was cut and weighed per F_1 family as an extra estimation of early vigour. Seedlings were transplanted into the field in late April 2012. Each plot contains 100 plants in a 10 x 10 layout with 15cm spacing and the distance between the plots was 1 m. From the early observations, some of the F_1 families appeared to outperform the parental cultivars. Further data collection is planned.

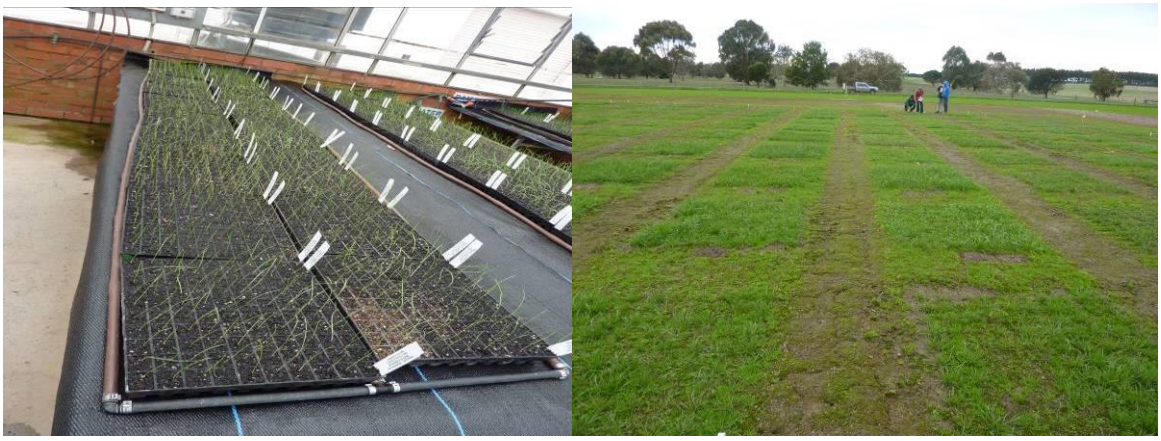


Figure 19. Seedling establishment and field-based establishment of the F_1 populations obtained from the Italian ryegrass crosses.

Perennial ryegrass

An advanced breeding scheme for cultivar sub-selection has been developed. The overall premise of the scheme is to select within a cultivar based on phenotypic performance or on associated genetic marker variation. The sub-selection, however, must maintain overall population frequencies to retain many of the initial cultivars characteristics.

Initial experimentation to validate this concept has been performed using data from the collection of pre-validated SNP markers that were screened across the clonal nursery germplasm. An assessment was made of the potential to select the *Lp*CAD2 SNP (described previously), that was predicted to account for c. 7% of V_p for DM digestibility. Two cultivars were identified as being amenable to improvement through sub-selection of plants. On inspection of SNP allele ratios within the cultivars, only 6 and 11% of individuals, respectively, in the two cultivars were identified as containing the beneficial allele. Selection of only this proportion of individuals would dramatically change the overall population frequencies, and significantly alter the overall performance of the synthesised cultivar. A collection of individuals were identified in the cultivars which would preserve the overall population allelic frequencies while selecting for the beneficial SNPs. This novel approach would lead to the fixation of a beneficial allele, while hopefully maintaining the initial desirable characteristics of the cultivar. The conservation of allele ratios in the initial cultivar would also allow direct

comparison of performance with the initial cultivar. The outcome of this breeding design is aimed at rapid improvement of currently elite cultivars in a specific trait, while maintaining background characteristics. The application is amenable to a range of phenotypic traits, higher success being likely for traits with larger heritabilities. These processes could be achieved rapidly and deliver sufficient seed quantity to assess cultivar stability over multi-site trials, which could in turn be fast-tracked as many characteristics of the original cultivars are likely being retained.

Specific plants from within the two cultivars that were identified as fulfilling these criteria were cloned and vernalised, and controlled polycrossing was performed. Significant seed yield was achieved enabling populations of seedlings to be established to validate the experimental approach. Based on predicted allele segregation from the crosses, c. 800 plants were germinated per cultivar. Each plant was genotyped for the marker allele of interest so that three groups per cultivar (homozygote, heterozygote and homozygote for the marker allele under selection) of c. 100 plants could be identified and established to generate spaced plant mini-swards for phenotypic assessment.

Additional crosses involving perennial ryegrass plants from the clonal nursery have been planned. These additional crosses use the same methodology as that employed with the Italian ryegrass plants, to preserve genetic diversity while selecting on the basis of phenotypic performance. Two specific subsets for crossing were identified, the first on the basis of biomass yield alone, and the second to select concurrently on the basis of biomass yield and WSC content. Parental ranges of phenotypic performance were derived along with an assessment of the range of diversity that has been selected (Figures 20 and 21). The target plants have been cloned and vernalised, then transferred to the glasshouse in which crossing is currently in progress.

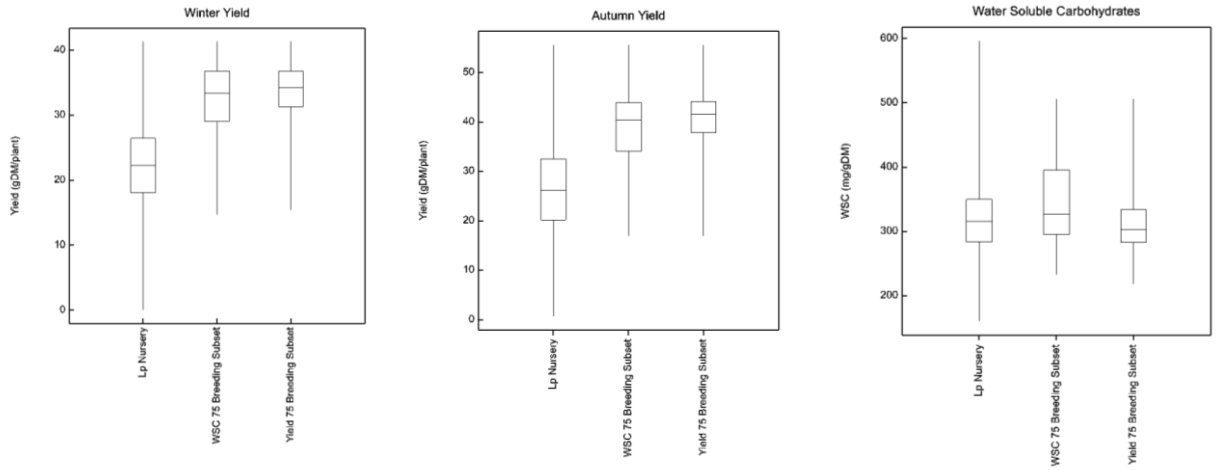


Figure 20. Box and whisker plots displaying the parental phenotypic range within the clonal nursery and the two selected sub-sets for crossing based on yield and WSC levels.

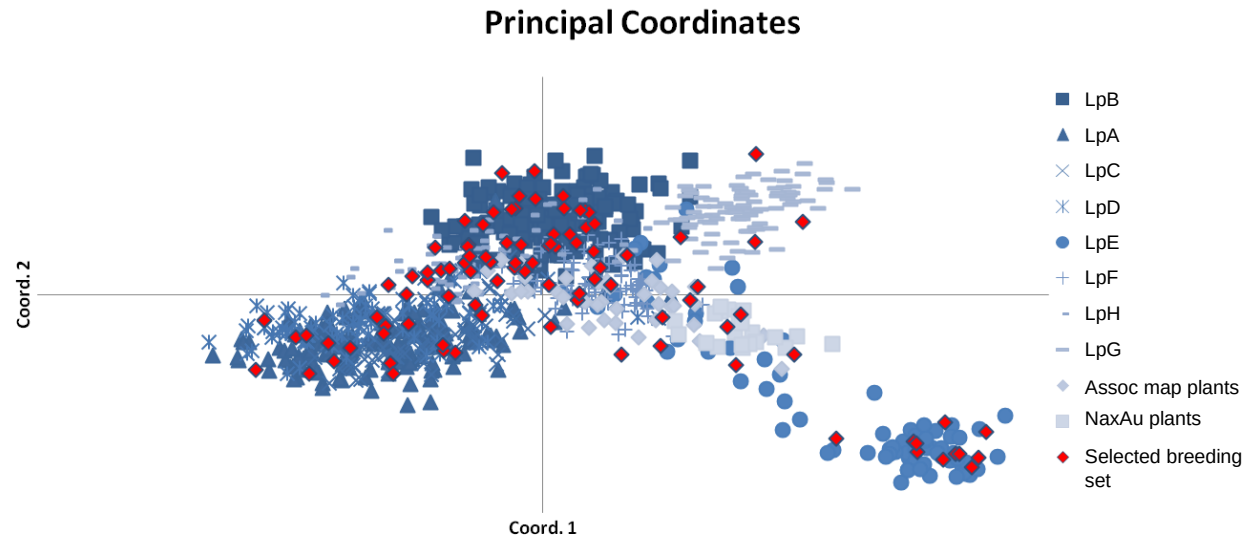


Figure 21. PCA distribution of the perennial ryegrass samples, individuals selected for crossing being identified to display the breadth of genetic diversity retained within the crossing scheme.

Extension of genotyping tools

Cultivar catalogue

Additional ryegrass cultivars have been supplied by the utilisation partner, encompassing a broader collection of currently available, relevant germplasm for the Australian dairy and red meat industries. Material includes examples of tetraploid perennial ryegrass and hybrid ryegrass cultivars, providing capacity to test applicability of the pre-formatted assay on an expanded variety of source material. These samples have been evaluated using the same collection of SNP markers as those used previously on the clonal nursery, which formed the basis of the pre-formatted assay.

The applicability of the SNP markers to identify tetraploid cultivars was experimentally tested. A collection of markers were identified that could distinguish a diploid sample from a tetraploid sample. The critical difference between diploid and tetraploid samples is the presence of additional genotypic classes. In the diploid sample, three genotypic classes are observed (AA, AB, BB) while in a tetraploid sample five are observed (AAAA, AAAB, AABB, ABBB, BBBB). The resolving power of the molecular markers must be sufficient to enable this distinction. Using the GoldenGate™ marker system, a cohort of 142 SNPs were identified that had sufficient resolving power to discriminate different ploidy levels within the tested samples tested (Figure 22).

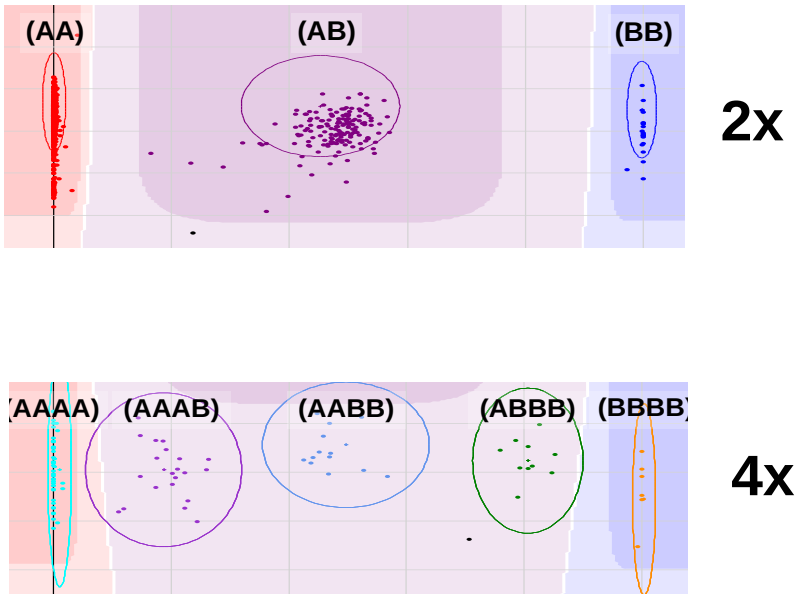


Figure 22. SNP 10012101 assessed across diploid (2x) and tetraploid (4) ryegrass samples. Each data point represents an individual ryegrass sample, genotypic classes being identified above the circled colour coded groups. Through comparison between the two images it can be seen that the tetraploid samples generate additional genotypic classes intermediate between the heterozygous and homozygous diploid classes.

Based on development of the StAMPP software package, data generated from the diploid and tetraploid cultivars could be processed together and significant differences identified. The previously identified cultivar relationships from assessment of the clonal nursery were all preserved, but with significant addition and enhanced resolution provided by the inclusion of the additional cultivars (Figure 23). The anticipated relationships between the tetraploid cultivars and their diploid counterparts were identified. The cultivars LpF and LpI are both marketed as having perennial ryegrass and meadow fescue parentage, but based on the current data and analysis methods they are not significantly different to other perennial ryegrass cultivars, and the issue of meadow fescue genetic introgression into this cultivar is under question. The cultivars LpE and LpM are both marketed as high in WSC, so it is unsurprising that they appear to have a close genetic relationship.

With regard to the Italian ryegrasses, no additional samples were genotyped and hence all of the previously identified relationships were preserved. The hybrid ryegrass cultivar LbA was included in the analysis (Figure 23), and was identified as more closely related to the Italian ryegrass germplasm that the perennial.

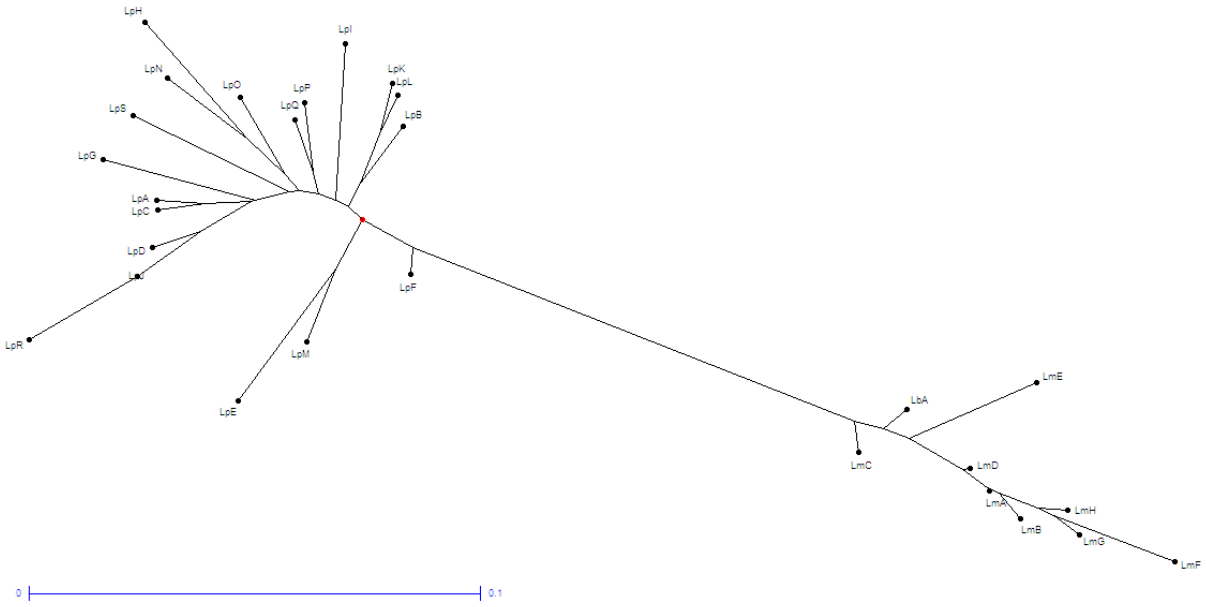


Figure 23. Perennial and Italian ryegrass cultivar relationships, depicting affinities between input germplasm used in the clonal nursery and an additional collection of cultivars selected for relevance to the farming community including an additional hybrid ryegrass cultivar (LbA).

Genetic profiling of cultivars has several outputs and applications. The ability to discriminate ryegrass cultivars will enable effective protection of plant breeders' rights (PBR), while also delivering to plant breeders the ability to make informed choices of germplasm to enter a breeding program based on genetic relationships as well as trial performance data. The cultivar catalogue will also assist in more transparent labelling and classification of cultivars. The current ryegrass classification system is based upon morphological characteristics as well as descriptions of breeding history, such that cultivars such as LpF and LpI are required to be described as hybrid ryegrass or a festulolium, although clearly belonging on genotypic criteria to the perennial ryegrass grouping. The cultivar catalogue will also describe the genetic relationships and pedigree of the newly released cultivars, so that varieties derived as sub-selections from existing cultivars can be readily identified and described as such. Development of the catalogue also delivers an enhanced understanding of molecular marker capabilities as well as, to date, the most comprehensive analysis of ryegrass genetics available to the Australian dairy farming community.

Genotyping-by-Sequencing SNP OPA

The development of the ryegrass 384-plex SNP OPA and cultivar catalogue was a significant step forward in understanding the genetic relationships between cultivars currently available to the Australian farming community, as well as assisting pasture plant breeders to combine genuinely different genetics by making more informed crossing decisions. However, the cost of entering a cultivar into the catalogue is a significant undertaking, and may have prevented wide adoption of the technology. An approach complementary to the initial SNP OPA procedure was developed. Due to the dramatic reduction in cost of DNA sequencing that has recently been achieved, a sequencing-based approach to SNP genotyping can be more cost-effective than more traditional methods.

The outcome of this protocol is that a batch of samples can be processed in a reduced or similar time frame (1-2 months) compared to the initial SNP OPA protocol, however the cost for performing the genotypic analysis has been reduced from c. $3,000-5,000 per sample to c. $400-600 per sample.

A second ryegrass varietal catalogue has been established as a result and is in the process of being significantly expanded beyond the scope of the original data set (Figures 24). The utilisation partners have indicated a significant interest in deploying the technology.

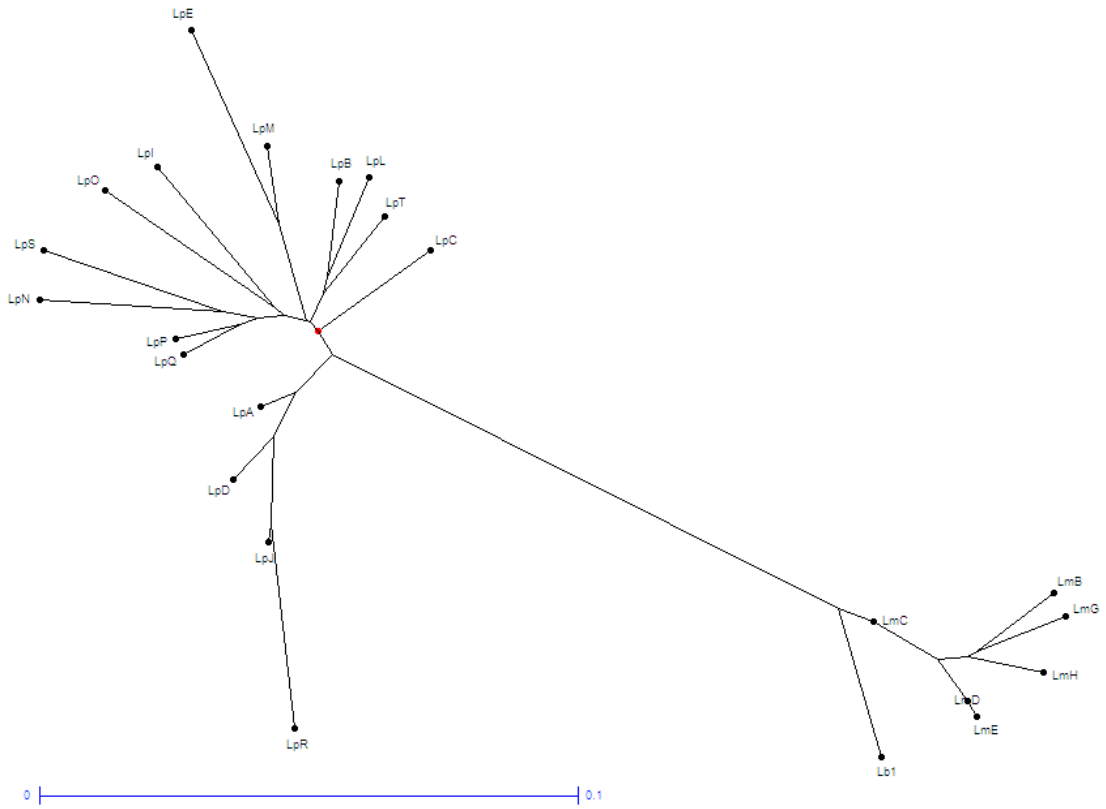


Figure 24. Perennial ryegrass and Italian ryegrass varietal relationships, determined through genotyping-by-sequencing implementation of the 384 SNPs from the OPA tool.

Endophyte genotypic analysis

Selected endophyte genotypes are deployed in newly synthesised varieties through inoculation of the shoot meristem region of individual young seedlings with mycelium grown on culture plates. The seed from which germinants are derived have had any resident endophyte previously removed. In skilled hands, efficient recovery rates are observed, such that multiple inoculated plants from each variety can be progressed into multiplication cycles, each maternal plant genotype contributing the inoculated endophyte strain to the next generation.

Previous studies in perennial ryegrass demonstrated that the frequency of inoculated endophytes can vary within and between varieties, potentially demonstrating host genotype-specific compatibility effects. In addition, the identity of an inoculated endophyte is dependent on accurate sampling of the cultured strain from a source plant, which may be compromised due to human error. Finally, adventitious presence of non-target endophytes may be observed during varietal development at multiple and largely unpredictable intervals, presumably due to seed admixture or maternal contributions from volunteer seedlings in field-based multiplication plots. The latter effect is generally associated with incursions of the standard toxic (ST) endophyte genotype, characteristic of the majority of germplasm derived from north-western Europe, and associated with high levels of the deleterious alkaloids lolitrem B and ergovaline. For all of these reasons, routine surveillance of endophyte identity and incidence during varietal development is critical for delivery of pasture grass varieties. The consequences of failure to detect anomalies would be delivery to the market either of varieties with low endophyte incidence, and hence limited persistence, or varieties with high adventitious ST presence, and hence toxicity problems for the grazing animal. These outcomes imply litigation risks for the genetic supply industry, and both productivity and animal health problems for the pastoral farmer.

Genetic purity testing for endophyte content has been performed as a routine exercise during the course of the project. In both years of the project, the utilisation partner has used the full allocation of the endophyte purity testing and has indicated a growing requirement for the future.

Year 1, 2010 - 2011
Plant and seed samples were obtained from the NZA seed multiplication process for commercial release. Incidences of unexpected endophyte presence were detected as well as incidence of low endophyte presence. Such issues with endophyte identity and stability represent significant issues. However, as a result NZA has repeated specific endophyte inoculations to remove any potential issues of poor quality product being delivered to the seed supply industry.

Year 2, 2011 - 2012
Plant and seed batches were submitted from the NZA seed multiplication process. The expected endophyte genotype was detected in the overwhelming majority of samples. However, in a several instances the presence of additional or alternative endophyte genotypes was detected. Examples where the additional or alternative endophyte was a desirable and commercially relevant strain and where the ST endophyte genotype were both present. In general, levels of endophyte presence were also high in the tested seed batches.

Integrated endophyte content and host analysis

Development of an integrated collection of SNP molecular markers to analyse host and endophyte has been performed. Specific SNP markers within the SNP-OPA tool have been experimentally tested, so that 4 SNP markers are present within the pre-formatted SNP-OPA tool that test for presence of endophyte DNA in the test sample as well as assessing variance within the endophyte genome to diagnose specific strains (figure 25). The strains that the test can diagnose are; AR1, NEA3, and ST endophyte, along with additional other non-*N.lolii* strains that will be available from NZA in coming years.

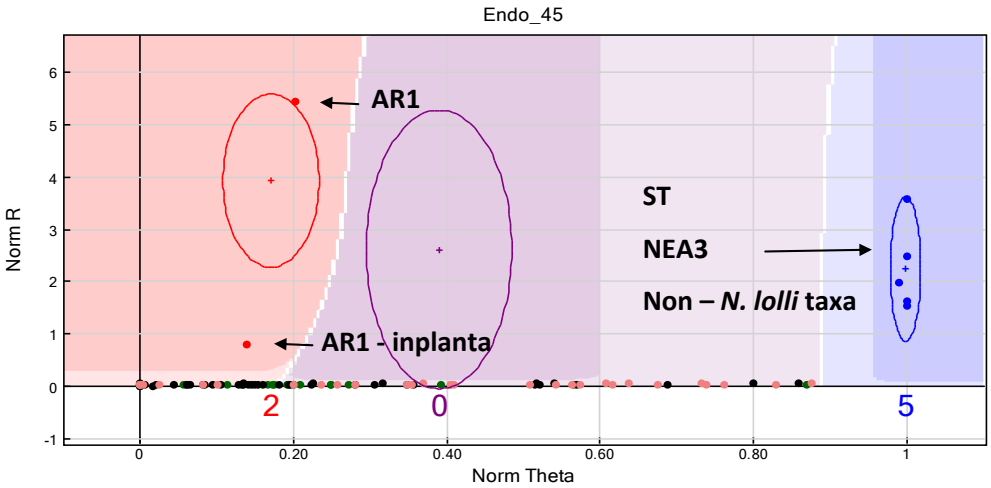


Figure 25. Demonstration of endophyte-specific SNP markers detecting and diagnosing the AR1 endophyte strain from the ST strain or the commercial NEA3 strain. Samples at the 0 value on the y-axis are endophyte free samples. The AR1 samples were tested as a DNA sample from a pure culture, as well as in planta-derived endophyte-positive sources.

A broader collection of endophyte strains and potential diagnostic SNPs have been assessed in the GBS-OPA. As the GBS-OPA uses a sequencing approach, regions of the endophyte genome that are higher in polymorphism rates are more ideally suited for strain identification and discrimination. The developed collection of SNPs includes all of the initial cohort of tests, but delivered a broader range of identified strains. From collaborative work within the Dairy Futures CRC, genomes of a large collection of endophyte strains have been sequenced, and two regions of the endophye genome were identified that were high in SNP frequency. Experimental work was undertaken to validate these new SNP loci and specific assays developed to be integrated within the GBS-OPA format to provide host and endophyte specific genotyping capacity within a single test as a complete assessment of the seed batch under investigation. The test for endophyte content and identity was implemented along with the development of the GBS-OPA varietal relationship matrix as previously described. The majority of samples provided were endophyte-negative, but one specific cultivar was provided with the strain AR1. The sample was processed and the data was analysed in parallel to all other samples (Figure 24). For each of the identified SNP bases from the endophyte, a minimum of 6489 and a maximum of 8045 sequence reads were aligned, and the specific bases present identified. On average 99.9% of the sequence reads were called for the reference base for the AR1 strain. The quality of the sequence generated has an accepted error rate of 0.1% explaining the missing reads. The GBS-OPA format permits alteration of the targeted SNP analysed, which enables migration of the assay to accommodate new novel endophytes as they become available and relevant.

5 Project outputs

From the experimental work performed to date, extensive analysis using detailed phenotypic and genotypic data has been performed for the clonal ryegrass plant nursery and associated sward trials. These project activities demonstrate a means of application for advanced phenotyping and genotyping in pasture plant breeding activities, and potential genetic gains to be obtained for on-farm benefit through improved feed-base quality and productivity.

A detailed catalogue of the genetic relationships between ryegrass cultivars has been generated that can establish the basis for a service of cultivar identification to assist in plant breeders rights. In addition, the cultivar catalogue describes the respective relationships between cultivars, which would provide knowledge to select novel and genuinely distinct germplasm as the feed-base for a farming system.

Application of endophyte QA/QC has directly identified levels of contamination in pre-commercial breeding lines of the industrial partner (NZA), allowing early intervention and elimination. As a direct result of the screening process applied in seed lots that were destined for seed multiplication, incidences of unexpected endophyte presence were detected at low levels and have bee removed. Through comparison with previous studies on the effect of different endophyte strains on milk production, ST endophyte typically reduces milk production by 2.7-35.2% when compared to low or no endophyte pasture, while non-toxic strains like AR1 are not significantly different[1]. This outcome supports one of the largest suppliers of high-performance ryegrass genetics to the Australian dairy industry to deliver their product with increased confidence for the benefit of the end-user, assuring animal health and safety, cultivar performance and improved pasture productivity

[1] Reviewed by I.J. Lean: Perennial ryegrass endophytes – effects on dairy cattle, Perennial ryegrass toxicosis in Australia, Published by Meat and Livestock Australia, North Sydney 2005

6 Recommendations

As a direct result from the activities performed within the described project several key recommendations for future directions and activities can be made:

- For the current utilisation partner, further development of genomics-assisted breeding technologies should be performed as a result of direct company investment.

- Interest from and potential uptake of technologies by additional utilisation partners to be assessed.

- Development and, more critically, ongoing maintenance with routine updates of a public ryegrass varietal catalogue should be performed that is comprehensive and freely available and could potentially be available from both websites of the funding organisations

- Application of the genotyping technology could enable testing of seed batches from the supply chain of seed sales for both cultivar identity and endophyte

identity, to classify incursions of foreign plant germplasm as well as standard toxic endophytes.

- Discussions with the relevant seed federations to describe the application of DNA profiling technology and its application in ryegrass species classification could be beneficial. This could improve and clarify the species description where currently many ryegrass species are misclassified due to specific morphological traits, which may result in confusion in the marketplace for the farming community.

- Under the circumstances of adoption and development of a national forage variety testing (NFVT) scheme, application of genotyping technology developed within the project, as well as high-throughput herbage quality assessment, could add significant value to the generated data.

- Development and assessment of high carbohydrate/low protein ryegrass for mitigation of greenhouse gases and assessment of nitrogen uptake or differences in fertilisation requirements could be beneficial.

- There are opportunities arising from the current project to explore options for future funding by the Gardiner Foundation in association with the MLA donor company in areas of common interest between the two organisations.

7 Project communication: Publications and presentations

Dairy and Red Meat Industry Communication

Laboratory tours and presentations have been provided to:

The Australian Association of Ruminant Nutritionalists
The University of Melbourne Dairy Systems Students
Chilean Dairy Technology Consortium
Dairy Futures CRC board
Large herd dairy farmers from the North and West of Victoria
Large herd dairy farmers from South Australia
Large herd dairy farmers from Tasmania
Large herd dairy farmers from Western Australia

Presentations were given by Prof. John Forster and associated project staff on 21st June 2011 at the Murray Dairy Young Dairy Development Program "Invite a Scientist to Lunch" at Shepparton.

Presentations were given by Prof. John Forster and associated project staff on 28th September 2011 at the South Gippsland Dairy Expo at Korumburra.

Presentation given by Prof. John Forster to the VDPI Meat and Wool Showcase, Melbourne on 8th May 2012.

In addition aspects of the project have been presented by Dr. David Nation to Fonterra Ltd (28th October 2010), Intelact (23rd June 2011), Dairy Food Safety Victoria (6th July 2011), Bega Cheese Ltd (8th February 2012), Australian Dairy Conference (23rd February 2012), Australian and New Zealand Co-operative leaders forum (17th April 2012), Horizon 2020 (29th May 2012)

Presentations were given by Drs. Wang and Cogan at the Dairy Futures CRC annual science forum on aspects of the project, in the presence of other scientific research groups and representatives from pasture plant breeding companies, the dairy processing industry as well as farming groups

Scientific Communications

Generic aspects of program activities to date were presented by Dr. Cogan to a scientific peer-group audience as part of the International *Lolium* Genome Initiative Workshop at international Plant and Animal Genome Conference XIX conference held in San Diego, California USA in January 2011.

Aspects of the program were also presented by Prof. Forster at the Seventh International Symposium on Molecular Breeding of Forage and Turf, held in Salt Lake City, Utah USA in June 2012. In addition 4 poster presentations were also given from related project activities.

8 Project personnel

Name	Substantive Role	Location
Prof. John Forster	State-Wide Leader and Principal Research Scientist (Molecular Genetics), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Bundoora
Prof. German Spangenberg	Executive Director, Biosciences Research Division, Victorian Department of Primary Industries	DPI-Bundoora
Dr. Noel Cogan	Senior Research Scientist (Molecular Genetics), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Bundoora
Dr. Junping Wang	Senior Research Scientist (Molecular Plant Breeding), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Hamilton
Dr Kathryn Guthridge	Senior Research Scientist (Molecular Genetics), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Bundoora
Dr Jatinder Kaur	Research Scientist (Molecular Genetics), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Bundoora
Michelle Drayton	Junior Research Scientist (Molecular Genetics), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Bundoora
Rebecca Baillie	Junior Research Scientist (Molecular Genetics), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Bundoora
Darren Pickett	Technical Assistant (Molecular Plant Breeding), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Hamilton
Russell Elton	Technical Assistant (Molecular Plant Breeding), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Hamilton
Carly Elliot	Technical Assistant (Molecular Plant Breeding), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Hamilton
Peter Hardy	Technical Assistant (Molecular Plant Breeding), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Hamilton
Tania Wilkinson	Technical Assistant (Molecular Plant Breeding), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Hamilton
Luke Pembleton	Ph.D. Student (Molecular Genetics), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Bundoora