Exploiting genetic diversity of forages to fulfil their economic and environmental roles

Proceedings of the 34th Meeting of
the EUCARPIA Fodder Crops and
Amenity Grasses Section
in cooperation with
the EUCARPIA Festulolium Working Group

Freising
6–8 September, 2021

Palacký University
Olomouc

Edited by S. Hartmann, S. Bachmann-Pfabe, S. Byrne, U. Feuerstein, B. Julier, R. Kölliker, D. Kopecky, I. Roldan-Ruiz, T. Ruttink, J.-P. Sampoux, B. Studer, T. Vleugels

Exploiting genetic diversity of forages to fulfil their economic and environmental roles

Proceedings of the 34th Meeting of
the EUCARPIA Fodder Crops and
Amenity Grasses Section
in cooperation with
the EUCARPIA Festulolium Working Group

Freising
6–8 September, 2021

Edited by
S. Hartmann
S. Bachmann-Pfabe
S. Byrne
U. Feuerstein
B. Julier
R. Kölliker
D. Kopecky
I. Roldan-Ruiz
T. Ruttink
J.-P. Sampoux
B. Studer
T. Vleugels

Palacký University
Olomouc

Edited by
Organising Committee of the 34th Meeting of the EUCARPIA Fodder Crops and Amenity Grasses Section,
Bavarian State Research Center for Agriculture, Vöttinger Straße 38, 85354 Freising, Germany

Editing and production by
Palacký University Press

Distributed by
Bavarian State Research Center for Agriculture
Institute for Crop Science and Plant Breeding
IPZ 4b: Forage Plant research – Breeding and Grassland Systems
Am Gereuth 4
85354 Freising, Germany
Klee-und-Grassamen@LfL.bayern.de

First edition

DOI: 10.5507/vup.21.24459677
ISBN 978-80-244-5967-7 (print)
ISBN 978-80-244-5968-4 (online: iPDF)
ISBN 978-80-244-5969-1 (online: ePub)

Organising Institutions

- Bavarian State Research Center for Agriculture (LfL)
- EUCARPIA Fodder Crops and Amenity Grasses Section
- EUCARPIA Festulolium Working Group

Organising Committee

Chairperson (LfL):	Stephan Hartmann	LfL
Chairperson (EUCARPIA):	David Kopecky	IEB
Member:	Silvia Bachman-Pfabe	IPK
	Christof Böhm	SZS
	Ulf Feuerstein	DSV
	Stefan Lütke Entrup	GfPi
	Wilbert Luesink	NPZ
	Mirko Rakoski	GfPi
	Bernhard Saal	PlantaServ
	Sabine Schulze	SZS
	Evelin Willner	IPK

Scientific Committee and Reviewers

Chairperson:	Stephan Hartmann	LfL
Members:	Silvia Bachmann-Pfabe	IPK
	Stephen Byrne	Teagasc
	Ulf Feuerstein	DSV
	Bernadette Julier	INRA
	Roland Kölliker	ETH
	David Kopecky	IEB
	Isabel Roldan-Ruiz	ILVO
	Tom Ruttink	ILVO
	Jean-Paul Sampoux	INRA
	Bruno Studer	ETH
	Tim Vleugels	ILVO

Opening address by the Bavarian State Minister for Food, Agriculture and Forestry Michaela Kaniber

I welcome all participants to the 34th meeting of the EUCARPIA Section "Fodder Crops and Amenity Grasses", which is now being organized by a German research institution again after almost two decades. We see the fact that this is being done by our Bavarian State Research Center for Agriculture (LfL) as a special recognition of our departmental research. Bavaria attaches particular importance to applied agricultural research, even when resources are scarce. In addition to the traditional state-run trials, which focus on production-related issues and in which the LfL plays a central coordinating role, Bavaria has also traditionally been engaged in breeding research by the LfL. This commitment is becoming increasingly important against the context of climate change and people's desire to reduce chemical plant protection, as breeding and breeding research is "the key technology" that can offer farmers solutions to face these changes. Forage grasses, clover and lucerne have a prominent position in this context. In most cases fodder plant seed is sown in mixtures, which alone makes a significant contribution to biodiversity in the field. Bavaria's farmers are already doing a great job here, unnoticed: they cultivate about one third of the total German area of clover, lucerne and their mixtures. Although clover, alfalfa and their mixtures are often associated with organic farming in the public perception because it is indispensable for it, 75% of Bavarian clover/alfalfa-grass is competitive in conventional crop rotations. More than one third of the German red clover multiplication is also located in Bavaria – although here more than 75% are managed by organic farmers. The topic of your meeting "Exploiting genetic diversity of forages to fulfil their economic and environmental roles" addresses precisely these aspects, the contributions shows the state of breeding research, but also the direction in which it must go in future in order to make the greatest possible impact as a key technology in plant production to the future questions of agriculture – not least that of Bavaria. I thank the members of the EUCARPIA Section "Fodder Crops and Amenity Grasses" for their multifaceted activities and wish the conference a successful proceeding.

Michaela Kaniber,
Bavarian State Minister for Food, Agriculture and Forestry

Preface

It is our great pleasure to welcome you to the 34th meeting of the EUCARPIA Fodder e Crops and Amenity Grasses Section. Due to the world-wide COVID-19 pandemic, our meeting will be held online.

This means that personal contacts are limited and participants will have no chance to explore experimental sites of the organizers, as well as historical downtown of Freising and Munich and its countryside and local peculiarities during conference excursions. That is a pity, – however, two sites one medal. Participating in the conference itself will be much easier without travelling hassle. The conference fees have been reduced to the amount that there is practically no cost-based barrier for participation. So we strongly believe that despite many challenges and constrains, the situation also brings new opportunities.

The general topic of the conference is 'Exploiting genetic diversity of forages to fulfil their economic and environmental roles' and a wealth of interesting contributions reflecting the diversity of forages and amenity grasses was submitted. We are very grateful for this, as success of each conference is tightly linked to the quality of the contributions. We hope that you will enjoy reading the papers as much as we did during the editorial and reviewing process. Altogether, 37 papers of invited and regular speakers enabled us to compile this book of Proceedings.

There are many talks about biodiversity in the social discourses, but people generally oversee one aspect, that is that in contrast to the breeding of wheat, maize, soybean and other arable crops, the breeding of forage plants deals with a large number of species which are frequently sown in mixtures. The most important forages are the grass species and the small-grain legumes (lucerne and clovers). In Bavaria, for example, state-recommended mixtures for grassland contain up to ten different species, and mixtures for field forage cultivation consist of up to eight species. In addition, fodder plant cultivars are genetically much broader because most of them are synthetics (populations), in contrast to wheat, maize and potato. Thus, in short: sowing of fodder plant seed is highly appreciated contributor to the biodiversity in crop rotation systems. Small-grain legumes are also responsible for a large part of the ecosystem services in crop rotation and are therefore indispensable for organic farming. In addition, they also serve as a source of diet for bees and other insects.

Climate change will affect areas that are disadvantaged in terms of crop production – and this usually includes permanent grassland (otherwise it would have been converted to arable land in the past) – more severely than good arable sites. Rapid adaptation to mitigate the impacts of climate change driven by breeding is essential to sustainably maintain the performance of the farms.

This EUCARPIA meeting reflects these important aspects and shows the state of current breeding and research of fodders and amenity grasses. Moreover, many contributions indicate the future directions of the breeding in tight connection with state-of-art technologies provided by international collaborative multidisciplinary approaches. This is prerequisite to provide the greatest possible contribution to the future issues of agriculture and crop breeding as a key technology in plant production.

All this is only possible through continuous research efforts and interdisciplinary collaboration of scientists and breeders in different research topics.

The meetings of the EUCARPIA Fodder Crops and Amenity Grasses Section are traditionally a marketplace of ideas where discussions are stimulated and often represent the starting point for new collaborations. We strongly believe that this meeting will be no exception.

We would like to thank all authors for submitting such a wide range of interesting contributions. We are grateful to all reviewers and editors for their critical feedback, which further improved the high quality of the manuscripts. Finally, we would like to express our gratitude to all members of the local organising committee and the scientific committee as well as many other colleagues and friends who supported us – and made this exciting symposium possible. We wish you an interesting scientific conference with many new insights and contacts and hope that you will also be curious about Freising with its long history, the campus with its scientific institutions, Bavaria with its diversity of landscape and culture and finally, and not least, Germany, which Bavaria is allowed to represent at this conference. We are looking forward and hope to meet you personally during future EUCARPIA meetings – or even in Weihenstephan/Freising – after the pandemic will be over.

Stephan Hartmann,
Bavarian State Research Center for Agriculture

Table of contents

Session 2: Characterizing genetic diversity – the basis for selection

Session 3: Strategies to optimally exploit genetic diversity

Session 4:
"Minor" and "new" species – solution for future challenges

Festulolium Workshop

DOI: 10.5507/vup.21.24459677.01

Reconciling the goals – grass breeding for an improved grassland husbandry

Johannes Isselstein and Martin Komainda

Department of Crop Sciences, Grassland Science, University of Göttingen, Germany, Von-Siebold-Str. 8, 37075 Göttingen

jissels@gwdg.de

Abstract. Grasslands of Europe are extremely divers. They can be grouped into different types and their management and resulting ecosystem services are quite different. To improve the overall performance of grasslands it is required to distinguish between the different grassland types and their potential delivery of ecosystem services. This holds for management measures as well as for forage plant breeding. The present papers analyses the potential contribution of germplasm improvement on the production and environmental function of grasslands and how this potential is determined by the different grassland types. While grassland husbandry is interested in beneficial traits of the grass sward as a whole, plant breeding is always centered around single species. Breeding progress in individual species must be assessed by the performance of the sward and the harvestable herbage. This includes that the performance of new germplasm has to be tested in species mixtures that are common in the farming practice. Apart from forage provision, environmentally important services such as carbon sequestration or biodiversity are becoming more important whereby changes in climate force to explore new candidate species that are not yet part of intensive breeding programs. New species will deliver traits with beneficial effects for grassland farming. Breeding will need to be more focused on bringing together species with complementary traits by selectively combining varieties of different species in a way to capture synergies.

Keywords: grassland types, ecosystem services, complementary traits, breeding for species mixtures.

1 Introduction

1.1 Grasslands and the services they provide

Grassland means land that is predominantly used by agriculture and that provides biomass with a high cell-wall content. The biomass is primarily utilized to feed ruminant livestock, but it can also be used for energy generation [1]. Apart from the provision of biomass of the grass sward, single plant species found

in grasslands have other functions as well, such as medicinal, food, hygienic/perfuming or ritual purpose [2]. These services are summarized as the production function of grasslands. However, grasslands also provide a range of non-production services; these concern the biodiversity, the climate, environmental pollution, the water and the cultural landscape [3]. The provision of this diversity of services has been named by the term multifunction grassland [4]. Essentially, it means that different targets of grassland management can be achieved simultaneously. Thus, a single objective should not be given priority at the expense of one or more other objectives [5].

European grassland are extremly diverse. This applies to the vegetation composition, the site and the management conditions of grassland. According to Allen et al. [6] and Peeters et al. [1], the following grassland types can be distinguished with regard to the age and persistence of the vegetation:

(i) *cultivated grassland*, usually sown or resown and regularly managed grassland, i.e. renovated, fertilized, weeds controlled, cultivated species more or less dominating,
(ii) *permanent grassland*, contain perennial or self-seeding forage species, persist indefinitely, sown species not or less dominating,
(iii) *temporary grassland/ley*, sown grassland composed of forage species, often integrated into arable crop rotations, sown species dominate,
(iv) *semi-natural grassland*, permanent grassland/habitat, vegetation dominated by indigenous and naturally occurring species.

The diversity of grassland causes considerable variation in the type and extent of services that can be provided by this agroecosystem. The consequence is that efficiency of resource use varies and that the use of yield-increasing and yield-securing inputs can have very different effects. If the performance of grassland multifunction is to be increased and grassland management improved, this particularity must be considered very carefully. This applies to management measures of grassland husbandry but also to plant breeding, which aims to change the traits of plant species in a way that required ecosystem services are better provided.

1.2 Objectives

The aim of this paper is to examine the role of forage plant breeding in improving the performance of grassland. In general, the demands on plant breeding of grassland are considerable. The objectives of grassland use are complex; in addition to the production function, environmental services have to be provided. These different objectives require partly different characteristics of the vegetation or plant species in use. Grassland occurs under extremely varying site and management conditions, species and varieties must therefore be adapted to a wide range of environmental conditions. The swards usually contain at least a few

but often even multiple species. New genotypes or varieties must therefore show their improved traits under conditions of intra- and interspecific competition in mixtures. Finally, the species must be able to maintain their improved trait properties in permanent swards for as many years as possible. For forage plant breeding the situation is complicated by the fact that breeding objectives concern the vegetative growth of the plants, whereas for seed production the generative development is decisive. In addition, forage plant breeding is much less profitable than cash crop breeding [7]. Moreover, breeding objectives are limited to a few specific traits [8], which is often not appropriate to capture the complexity of grassland farming. Against this background, it is particularly important to define objectives of forage plant breeding precisely according to grassland types and to align them with the challenges that grassland farming is facing.

This article first describes a differentiated evaluation of ecosystem services that are expected from grassland according to grassland types. Based on this, grassland management measures that contribute to improving the services are analysed. Since plant breeding is to be regarded as a decisive path for the improvement of grassland, special attention is paid to the extent to which the services can be increased through breeding improvements in forage plant species.

2 Targets of an Improved Grassland Husbandry

In order to be able to identify the need for improving grassland management, it is first necessary to determine the priorities in ecosystem services depending on the type of grassland. In Table 1, a ranking has been made in relation to the importance of different ecosystem services based on existing studies. In cultivated and temporary grassland, forage production has priority; in permanent and semi-natural grassland, biodiversity and environmental services are partly more important than the production function. This has consequences for measures to improve grassland management and in particular for the use of genetic progress in forage plant species. In permanent and semi-natural grasslands, the vegetation is usually not sown and can be considered as a result of the specific site and management conditions. Accordingly, breeding does not play a role here. On the contrary, the introduction of improved genotypes would even compromise the goal of preserving diversity and genetic resources. The situation is different for cultivated and temporary grassland. Through regular sowing and sward renovation, new genotypes can be introduced into the swards, so the vegetation is more or less controlled and modified in a beneficial way chosen to meet the desired aims. The breeding progress can thus become directly effective.

Table 1. Importance of ecosystem services provided from grasslands depending on the grassland type.

Ecosystem service	Cultivated grassland	Permanent grassland	temporary grassland	semi-natural grassland
Herbage provision	+++*)	++	+++	+
Soil-C/C-sequestration	++	+++	+	+
Nitrogen emission	++	++	++	+
Water provision	++	+++	++	++
Biodiv./Genet. resources	+	++	-	+++

*) importance/priority: '+++' highest, '++' moderate to high, '+' low, '-' no

In order to provide the required ecosystem services of grassland, measures in grassland management are taken that are initially focused on the functionality of the sward as a whole community and less on the performance of individual forage plant species. Therefore, the performance of grassland is assessed on the basis of the traits present in the swards. In terms of forage provision, these are the following traits: (i) herbage productivity, i.e. growth potential, regrowth capacity after defoliation or after a period of inhibited growth, uniform seasonal pattern of production, drought tolerance, long-term persistence, sward density and tolerance or resistance to diseases and pests. (ii) forage quality, i.e. the concentration of nutrients relevant for animal nutrition, the palatability of the forage, and the digestibility or metabolisability of the nutrients into animal performance. With regard to the ecosystem services of carbon sequestration, environmental protection and water supply, these are properties such as root growth, litter degradability, and the appropriation uptake and utilisation efficiency for water and nutrients. This raises the question of the extent to which these properties of the sward can be supported by plant breeding.

3 The Role of Forage Grass Breeding

Forage plant breeding is expected to play a greater role in improving grassland performance in the future. Increases in performance will no longer be achieved by further increases in inputs, but by increases in efficiency that enable the maintenance of performance with reduced inputs [9]. The potential performance of the sward is shaped by its vegetation composition. In general, sward improvement can be addressed in two ways: (i) species selection and composition, and (ii) varietal selection within a forage species. Breeding work starts with species selection, the actual genetic improvement is then done by developing varieties within the species.

The range of forage plants cultivated by breeding is small compared to the range of species found in semi-natural or permanent grassland. By expanding the range and adding new species to the sward, the traits of the sward can be significantly changed in the short term. Examples of this are hitherto little-noticed

forage plant species that contain specific secondary metabolites. These can exert positive effects on the nutrient use efficiency in the digestive tract of the livestock [10]. This refers to a reduced excretion of nitrogen in urine, or the reduction of enteric methane emission. Referring species include dicot plants such as minor legumes or plantain. The same applies to the drought tolerance of swards. The variability within common grass species such as perennial ryegrass is not sufficient to adequately maintain forage production under increasing incidence of periodic drought [11] making the introduction of new species with an inherently higher drought tolerance meaningful. From a grassland management perspective, it would be desirable to broaden the range of species to be sown and to improve these new species in important forage production properties through breeding.

For the future of forage plant breeding there is a need to better consider traits that support the non-production functions of grasslands, in addition to the production function. These include, above all, the reduction of emissions of greenhouse gases, the reduction of environmentally harmful nutrient losses or the increased accumulation of carbon in the soil.

In the course of forage plant breeding, the traits of single species are improved. Thereby, it is not clear to what extent improved traits become visible in species mixtures that are common in the farming practice. In mixtures, different plant species interact, there is competition for growth factors and there are also complementary effects. The expression of species-specific traits can thus be more or less effective. In recent years, there have been increasing efforts to specifically combine forage plant mixtures in order to maximise the complementarity of different species. It is necessary to examine to what extent breeding of individual species can take into account the suitability of a new variety for such 'designer mixtures'.

4 Conclusions

Grassland farming is facing considerable challenges as, in addition to the production of high-quality biomass, the provision of environmental services is increasingly required. Forage plant breeding can play an important role in meeting these challenges. This requires that the multiple objectives of grassland management are precisely identified and aligned with the potential for improvement in plant breeding. There are new breeding objectives that appear to make a general expansion of the spectrum of species being bred necessary. In addition, breeding objectives are becoming increasingly complex. Desired properties of the sward will not be achieved by improving a single species. Rather, breeding will be more focused on bringing together important characteristics by selectively combining varieties of different species with complementary traits to balance interspecific competition in a way to capture synergies. This will change both breeding methods and testing procedures for variety approval.

References

[1] Peeters, A., Beaufoy, G., Canals, R.M., De Vliegher, A., Huyghe, C., Isselstein, J., Jones, G., Kessler, W., Kirilov, A., Mosquera-Losada, M.R., Nilsdotter-Linde, N., Parente, G., Peyraud, J.-L., Pickert, J., Plantureux, S., Porqueddu, C., Rataj, D., Stypinski, P., Tonn, B., van den Pol – van Dasselaar, A., Vintu, V., Wilkins, R.J. (2014): Grassland term definitions and classifications adapted to the diversity of European grassland-based systems. Grassland Science in Europe 19, 743–750.

[2] Gemedo-Dalle, T., Maass, B.L., Isselstein, J. (2005): Plant biodiversity and ethnobotany of Borana pastoralists in Southern Oromia, Ethiopia. Economic Botany 59, 43–65.

[3] Isselstein, J., Kayser, M. (2014): Functions of Grassland and their potential in delivering ecosystem services. In: Hopkins A., Collins R.P., Fraser M.D., Kind V.R., Lloyd D.C., Moorby J.M., Robson P.R.H. (eds) The Future of European Grasslands. Grassland Science in Europe 19, 199–203.

[4] Lemaire, G., Wilkins, R., Hodgson, J. (2005): Challenges for grassland science: managing research priorities. Agriculture, Ecosystems & Environment 108, 99–108.

[5] Isselstein, J. (2021): Towards an informed grassland farming – Shortfalls and perspectives. Grassland Science in Europe 26, 2–10.

[6] Allen V.G., Batello C., Berretta E.J., Hodgson J., Kothmann M., Li X., McIvor J., Milne J., Morris C., Peeters A. and Sanderson M. (2011) An international terminology for grazing lands and grazing animals. Grass and Forage Science 66, 2–28.

[7] Annicchiarico, P., Boller, B., Brummer, E.C., Reheul, D.: Improving the focus of forage breeding research. In: Roldán-Ruiz, I. et al. (eds.), Breeding in a World of Scarcity: Proceedings of the 2015 Meeting of the Section "Forage Crops and Amenity Grasses" of Eucarpia, 251–269 (2016).

[8] Capstaff, N.M., Miller, A.J., 2018: Improving yield and nutritional quality of forage crops Frontiers in Plant Science 9, 535, pp.1–18 (2018).

[9] Parsons, A.J., Edwards, G.R., Newton, P.C.D., Chapman, D.F., Caradus, J.R., Rasmussen, S., Rowarth, J.S.: Past lessons and future prospects: Plant breeding for yield and persistence in cool-temperate pastures. Grass and Forage Science 66, 153–172 (2011).

[10] Kapp-Bitter, A.N., Dickhoefer, U., Kreuzer, M., Leiber, F.: Mature herbs as sup-plements to ruminant diets: effects on in vitro ruminal fermentation and ammonia production. Animal Production Science 61, 470–479, (2021).

[11] Hofer, D., Suter, M., Haughey, E., Finn, J.A., Hoekstra, N.J., Buchmann, N., Lüscher, A.: Yield of temperate forage grassland species is either largely resistant or resistant to experimental summer drought. Journal of Applied Ecology 53, 1023–1034, (2016).

Session 1:
Natural diversity – a valuable source for breeding

 DOI: 10.5507/vup.21.24459677.02

Making the most of multi-species mixtures: the role of species and functional diversity in intensively managed grasslands

Caroline Brophy[1], Guylain Grange[1,2], Carsten Malisch[3] and John Finn[2]

[1] Trinity College Dublin, Dublin 2, Ireland
[2] Teagasc, Johnstown Castle, Wexford, Ireland
[3] Christian-Albrechts-University, Kiel, Germany

caroline.brophy@tcd.ie

Abstract. Intensively managed grasslands with low species diversity and high inorganic nitrogen fertiliser regimes can be economically and environmentally expensive. Multispecies mixtures have the potential to provide solutions to improve the sustainability of productive systems. We review landmark studies that investigate the role of species and functional group diversity, and extreme weather events, on intensively managed grassland outcomes. We also introduce a new global network of experiments (LegacyNet) investigating the role of multispecies grassland mixtures within crop rotations. Multi-site experiments across wide spatial gradients help to generalise ecological results and can identify climatic zones that may benefit from local selection of species and cultivars to facilitate persistence and optimise the performance of each functional group in multispecies mixtures.

Keywords: grass, legume, herb, species diversity, sustainable solutions.

1 Introduction

Intensively managed grasslands across large areas of Europe have traditionally been characterised by single species (monoculture) pastures, while intensive arable pastures are often characterised by monocropping (continuous monoculture), both of which require high inorganic nitrogen fertiliser inputs [1, 2]. These productive systems are associated with negative environmental outcomes, such as high greenhouse gas emissions. Evidence is mounting that increased species diversity may provide solutions to improve sustainability in agroecosystems, while maintaining high productivity [3].In this paper, we review landmark studies investigating the role of species and functional diversity in intensively managed grasslands over the past 15 years, including investigations into the potential of multispecies mixtures to mitigate extreme weather events. We also introduce

a new global network of experiments called LegacyNet. Finally, for intensively managed multispecies grasslands, we highlight potential research goals within plant breeding programmes to achieve sustainability goals in agroecosystems.

2 Methods

Here we describe three groups of experimental studies on intensively managed grassland systems: (1) The Agrodiversity Experiment which was completed in the late 2000's; (2) a recent set of experiments that manipulated drought conditions; and (3) a new ongoing global network of field experiments called LegacyNet. Measurements recorded across these studies include harvested total annual dry matter yield, weed invasion, and forage quality.

(1) In the Agrodiversity Experiment, a common experiment was implemented across 31 pan-European sites [4]. At each site, two grasses and two legumes were manipulated across a diversity gradient, including monocultures and four-species mixtures of varying evenness, and repeated at two sowing densities. A total of 30 plots per site were measured over a three-year period.

(2) In a two-site experiment (in Ireland and Switzerland) one grass, one herb and two legume species were manipulated across a diversity gradient under rainfed and drought conditions, with a total of 70 plots at each site measured over two years [5, 6]. A subsequent experiment was established (in Ireland) where two grass, two legume and two herb species were manipulated across a species diversity gradient from monocultures up to six-species mixtures under rainfed and drought conditions, with a total of 86 plots measured over two years [7].

(3) A new network of experiments called LegacyNet has been established spanning five continents and across a wide range of climatic conditions; the LegacyNet website is https://legacynet.scss.tcd.ie/ (accessed June 2021). At each site two grass, two legume and two herb species are established across a diversity gradient from monocultures to six-species mixtures in a total of 52 plots. There are approx. 30 sites (so far) established in the network. The grassland ley plots will be measured for at least 18 months, at which stage a follow-on crop will be established (replacing the grassland ley in each plot), managed uniformly across plots, and measured for a full growing season. We will investigate the effect of the grassland ley diversity gradient on both the grassland ley outcomes, and on the follow-on crop performance.

3 Results

Positive effects of four-species grass-legume mixtures over monocultures were shown for biomass yield [8, 9] and weed invasion [10] across the 31 sites of the Agrodiversity Experiment. Transgressive overyielding was evident at most sites, with yields of four-species mixtures generally outperforming the best performing

monoculture [8]. Changes in species relative abundances over the three years were driven by relative growth rate differences of the competing species [11]. However, even when legumes declined over time, yield benefits from mixing four species persisted, albeit, at a reduced rate [11].

Increasing species richness (up to four species) and functional trait diversity (up to three functional groups: grass, legume and herb) improved yield stability under an experimentally imposed drought [5, 6], while six species mixtures with two grasses, two legumes and two herbs, were more resilient to drought compared to monocultures [7].

Preliminary analysis of data from a single Irish site of the LegacyNet experiment indicates strong potential of mixing three functional groups to benefit multiple agronomic and environmental ecosystem functions (yield, N yield, emissions). Modelling the variation of species- and functional-group-specific effects from site to site across the LegacyNet sites will help to identify climatic conditions where individual species or functional groups may benefit from local selection or investment in plant breeding to produce improved locally adapted species.

4 Conclusion and Discussion

The evidence is strong that mixing four species in two functional groups (grass and legume) will improve the outcomes in intensively managed grasslands, reduce inorganic fertiliser requirements and help protect against extreme drought events [5–10]. There is also emerging evidence promoting the inclusion of herb species [5–7, 12]. Persistence of individual species or functional groups may present agronomic management challenges since relative competitive abilities of selected species may lead to reduced diversity over time [11]. A major benefit of multi-site experimentation, such as the Agrodiversity Experiment and LegacyNet, is to identify climatic regions where cultivar selection and breeding may facilitate the maintenance of diversity in multispecies mixtures over desired timeframes. Such experiments provide insights for breeders about the design and sustainability of multi-species mixtures in intensively managed grasslands.

References

[1] Lüscher, A., J.A. Finn, J. Connolly, M.T. Sebastia, R.P. Collins, M. Fothergill, C. Porqueddu, C. Brophy, O. Huguenin-elie, et al (2008): Benefits of sward diversity for agricultural grasslands. Biodiversity, 9: 29–32.

[2] Lemaire, G., F. Gastal, A. Franzluebbers, and A. Chabbi (2015): Grassland–cropping rotations: an avenue for agricultural diversification to reconcile high production with environmental quality. Environmental Management, 56(5): 1065–1077.

[3] Isbell, F., P.R. Adler, N. Eisenhauer, D. Fornara, K. Kimmel, C. Kremen, D.K. Letourneau, M. Liebman, H.W. Polley, et al. (2017): Benefits of increasing plant diversity in sustainable agroecosystems. Journal of Ecology, 105(4): 871–879.

[4] Kirwan, L., J. Connolly, C. Brophy, O. Baadshaug, G. Belanger, A. Black, T. Carnus, R. Collins, J. Čop, et al. (2014): The Agrodiversity Experiment: three years of data from a multisite study in intensively managed grasslands. Ecology, 95(9): 2680.

[5] Haughey, E., M. Suter, D. Hofer, N.J. Hoekstra, J.C. McElwain , A. Lüscher, and J.A. Finn (2018): Higher species richness enhances yield stability in intensively managed grasslands with experimental disturbance. Scientific Reports, 8: 15047.

[6] Hofer, D., M. Suter, E. Haughey, J.A. Finn, N.J. Hoekstra, N. Buchmann, and A. Luscher (2016): Yield of temperate forage grassland species is either largely resistant or resilient to experimental summer drought. Journal of Applied Ecology, 53(4): 1023–1034.

[7] Grange, G., J. Finn, and C. Brophy (2021): Plant diversity enhanced yield and mitigated drought impacts in intensively managed grassland communities. Journal of Applied Ecology, in press.

[8] Finn, J.A., L. Kirwan, J. Connolly, M.T. Sebastia, A. Helgadottir, O.H. Baadshaug, G. Belanger, A. Black, C. Brophy, et al. (2013): Ecosystem function enhanced by combining four functional types of plant species in intensively managed grassland mixtures: a 3-year continental-scale field experiment. Journal of Applied Ecology, 50(2): 365–375.

[9] Kirwan, L., A. Lüscher, M.T. Sebastià, J.A. Finn, R.P. Collins, C. Porqueddu, A. Helgadóttir, O.H. Baadshaug, C. Brophy, et al. (2007): Evenness drives consistent diversity effects in intensive grassland systems across 28 European sites. Journal of Ecology, 95(3): 530–539.

[10] Connolly, J., M.T. Sebastia, L. Kirwan, J.A. Finn, R. Llurba, M. Suter, R.P. Collins, C. Porqueddu, A. Helgadottir, et al. (2018): Weed suppression greatly increased by plant diversity in intensively managed grasslands: A continental-scale experiment. Journal of Applied Ecology, 55(2): 852–862.

[11] Brophy, C., J.A. Finn, A. Lüscher, M. Suter, L. Kirwan, M.-T. Sebastià, Á. Helgadóttir, O.H. Baadshaug, G. Bélanger, et al. (2017): Major shifts in species' relative abundance in grassland mixtures alongside positive effects of species diversity in yield: a continental-scale experiment. Journal of Ecology, 105(5): 1210–1222.

[12] Cong, W.F., M. Suter, A. Lüscher, and J. Eriksen (2018): Species interactions between forbs and grass-clover contribute to yield gains and weed suppression in forage grassland mixtures. Agriculture, Ecosystems & Environment 268: 154–161.

 DOI: 10.5507/vup.21.24459677.03

Data management for preserving genetic diversity: Experiences and challenges

Stephan Weise[1][0000-0003-4031-9131]

[1] Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben, Corrensstr. 3, 06466 Seeland, Germany

weise@ipk-gatersleben.de

Abstract. Genebanks play an important role in the preservation of global biodiversity. Here, more than seven million samples are conserved *ex situ* worldwide. Well-structured documentation of all associated data and information is the basic prerequisite for the operation of genebanks. This makes it possible to unlock the treasures stored in them for research and breeding. The background to the documentation of genebank collections is presented and cooperation at international level is described. In addition, challenges facing documentation are illustrated.

Keywords: Plant genetic resources, genebank, documentation, information system.

1 Background

Genebanks make an important contribution to the long-term conservation of plant genetic resours [9]. They complement the conservation of biodiversity in farmers' fields and in nature. There are approximately 1,800 genebanks worldwide, of which more than 600 are located in Europe [4]. Apart from preserving the physical samples, data management is one of the most important tasks of a genebank. At the same time, it is also one of the greatest challenges [17] [5] [6]. Worldwide, more than seven million accessions are maintained *ex situ* in genebanks. In order to be able to make statements about the potential value of these resources, as much information as possible must be gathered. A well-structured documentation of all data and information available on a genebank accession is therefore the basic prerequisite for the use of genebanks. Against this background, a broad spectrum of data must be considered:

- Data that allow the value of the genetic resources to be assessed
  - Passport data: These data are elementary and provide information on taxonomy, type of material, collecting sites, sources of acquisition, etc. [1].

  - Phenotypic observations: These data are used to describe structural and functional traits of plants and to make statements about e.g. susceptibility to diseases, drought stress, etc. [11].
  - Comprehensive genetic data: These data make it possible to better unlock the biodiversity preserved in genebanks [15].
- Pure management data such as germination rates of samples, storage locations, health tests or responsibilities for conservation.
- Information of legal relevance such as collecting permits, correspondence with donors or material transfer agreements.

Moreover, it should not be forgotten that the tasks of genebanks are very long-term. Both the focus of the documentation and the technologies used for it are constantly evolving. In addition, organisational changes as well as personnel changes occur. Nevertheless, continuous documentation must be ensured.

2 Data Management

Starting with the use of index cards and field books, there was a gradual development towards electronic data management [10]. However, the resulting information systems remained largely isolated from each other. This did not change until the beginning of the 1980s, when data on accessions of one or more species maintained in different genebanks began to be compiled. Thus, the concept of Central Crop Databases (CCDBs) was born [8].

The aim of the Central Crop Databases was to strengthen cooperation between genebanks, to improve the availability of genebank material to users and to identify potential duplicates between genebanks. However, it must be stated that these goals were only partially achieved. The availability of these databases was limited and the quality of the data provided was often too low [14]. Uniform standards for the description and exchange of passport data were also lacking. Subsequently, this led to the development of the Multi-Crop Passport Descriptors (MCPD), which have now become a globally accepted standard [2] [1]. This and other standards, such as Darwin Core [19], form the nucleus for aggregator systems that enable searches across genebanks. Systems such as WIEWS[1], EURISCO[2], Genesys[3] and GBIF[4] should be mentioned here in particular. The European approach will be further described in more detail.

The European Search Catalogue for Plant Genetic Resources (EURISCO) is an accession-level information system, which provides detailed information

[1] World Information and Early Warning System on Plant Genetic Resources for Food and Agriculture (WIEWS) http://www.fao.org/wiews/, last accessed 2021-05-28

[2] European Search Catalogue for Plant Genetic Resources (EURISCO), http://eurisco.ecpgr.org/, last accessed 2021-05-28

[3] Genesys, https://www.genesys-pgr.org/, last accessed 2021-05-28

[4] Global Biodiversity Information Facility (GBIF), https://www.gbif.org/, last accessed 2021-05-28

on the majority of European *ex situ* collections of plant genetic resources [18]. About 400 of the European collections regularly provide passport data on more than two million accessions, covering more than 6,700 genera and 45,000 species. EURISCO has been developed since 1999 and is operated within the framework of the European Cooperative Programme for Plant Genetic Resources (ECPGR). The system is based on a network of National Inventories from 43 member countries. In addition to passport data based on the MCPD standard, EURISCO increasingly provides phenotypic observation data. EURISCO thus provides a central entry point for data on European plant genetic resources collections, which can be searched uniformly across genebanks via a web interface.

3 Challenges

Regardless of the great progress that has been made in this area in recent decades, a number of challenges remain. A selection of these will be mentioned below as examples.

As mentioned above, the MCPD standard for describing and exchanging passport data between plant genebanks is well established. Nevertheless, the quality and quantity of the data often still leave room for improvement. A frequently occurring problem in this context is incorrect coordinate information. This requires continuous data curation efforts. Another challenge is the use of synonymous taxonomic terms. The genebanks that exist worldwide sometimes follow different taxonomic schools, opinions or traditions. Although this is both syntactically and semantically correct, it makes it difficult for users to find suitable genebank accessions, as extensive background knowledge is required for searches. However, by mapping against public taxonomic repositories, it is possible to include a wide range of synonymous terms in searches [12].

Another major challenge is the unique identification of genebank accessions because many genebanks make changes to accession identifiers in the course of time. By exchanging material with other genebanks, breeders and researchers, this creates chains of identifiers that are difficult to track over time [7]. This poses a particular challenge for the aggregator systems described above. In 2015, the Secretariat of the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) therefore advocated the use of Digital Object Identifiers (DOIs) as unique identifiers for genebank accessions and provided a corresponding infrastructure for the allocation of DOIs. DOIs have now established themselves as a quasi-standard for the unique identification of plant genetic resources [3].

One difficulty associated with the previous point is the clear identification of material derived from a genebank accession or, more accurately, the clear distinction from the original accession. The genotyping of entire genebank collections, which is increasingly being carried out, is done on the basis of SSD lines [13]. Unfortunately, it still happens regularly that these are treated as equivalent to the

original accessions, although they might only represent a section of them. This must be well documented [17].

The last challenge to be mentioned here concerns phenotypic observation data. Unlike passport data, there are no widely accepted standards for this. There is a wide variety of trait names and synonymous terms as well as different rating scales. In addition, the description of experiments is often not uniform. This makes it very difficult to compare data collected in different experiments or even at different institutions. No generally accepted solution is yet in sight, but there are approaches such as MIAPPE [11] that at least aim to harmonise experiment descriptions.

4 Summary and Conclusion

The management of data on genebank accessions is an indispensable basic requirement for the conservation and development of these valuable resources. In addition to describing the material as completely as possible, this includes a whole range of other information. The cross-genebank cooperation in the field of documentation has made an important contribution to the standardisation of these data. In this context, the important role of regional and international networks such as the ECPGR should be emphasised, which make it possible to pool existing strengths and resources.

References

[1] Alercia A, Diulgheroff S, Mackay M (2015): FAO/Bioversity Multi-Crop Passport Descriptors V.2.1 (MCPD V.2.1). Food and Agriculture Organization of the United Nations (FAO), Rome (Italy); Bioversity International, Rome (Italy),

[2] Alercia A, Diulgheroff S, Metz T (2001): FAO/IPGRI Multi-Crop Passport Descriptors (MCPD). Food and Agriculture Organization of the United Nations (FAO), Rome (Italy); International Plant Genetic Resources Instit., (IPGRI), Rome (Italy),

[3] Alercia A, López FM, Sackville Hamilton NR, Marsella M (2018): Digital Object Identifiers for food crops – Descriptors and guidelines of the Global Information System. Food and Agriculture Organization of the United Nations, Rome

[4] Engels JMM, Maggioni L (2012): AEGIS: a regionally based approach to PGR conservation. In: Maxted N, Dulloo ME, Ford-Lloyd BV, Frese L, Iriondo JM, Pinheiro de Carvalho MAA (eds) Agrobiodiversity conservation: securing the diversity of crop wild relatives and landraces. CABI, pp 321–326. doi:10.1079/9781845938512.0321

[5] FAO (2010): The Second Report on the State of the World's Plant Genetic Resources for Food and Agriculture. Commission on Genetic Resources for Food and Agriculture, Food and Agriculture Organization of the United Nations, Rome

[6] Fowler C, Hodgkin T (2004): Plant Genetic Resources for Food and Agriculture: Assessing Global Availability. Annual Review of Environment and Resources 29 (1):143–179. doi:10.1146/annurev.energy.29.062403.102203

[7] Garrity GM, Thompson LM, Ussery DW, Paskin N, Baker D, Desmeth P, Schindel DE, Ong PS (2009): Studies on Monitoring and Tracking Genetic Resources. United Nations Convention on Biological Diversity, Report No. UNEP/CBD/WG-ABS/7/INF/2, Rome

[8] Gass T, Lipman E, Maggioni L (1997): The role of Central Crop Databases in the European Cooperative Programme for Crop Genetic Resources Networks (ECP/GR). Paper presented at the Central Crop Databases: Tools for Plant Genetic Resources Management,

[9] Hoisington D, Khairallah M, Reeves T, Ribaut J-M, Skovmand B, Taba S, Warburton M (1999): Plant genetic resources: What can they contribute toward increased crop productivity? Proceedings of the National Academy of Sciences 96 (11):5937–5943. doi:10.1073/pnas.96.11.5937

[10] Knüpffer H (1983): Computer in Genbanken – eine Übersicht. Die Kulturpflanze 31 (1):77–143. doi:10.1007/BF02000699

[11] Krajewski P, Chen D, Ćwiek H, van Dijk ADJ, Fiorani F, Kersey P, Klukas C, Lange M, Markiewicz A, Nap JP, van Oeveren J, Pommier C, Scholz U, van Schriek M, Usadel B, Weise S (2015): Towards recommendations for metadata and data handling in plant phenotyping. Journal of Experimental Botany 66 (18):5417–5427. doi:10.1093/jxb/erv271

[12] Kreide S, Oppermann M, Weise S (2019): Advancement of taxonomic searches in the European search catalogue for plant genetic resources. Plant Genetic Resources: Characterization and Utilization 17 (6):559–561. doi:10.1017/S1479262119000339

[13] Milner SG, Jost M, Taketa S, Mazón ER, Himmelbach A, Oppermann M, Weise S, Knüpffer H, Basterrechea M, König P, Schüler D, Sharma R, Pasam RK, Rutten T, Guo G, Xu D, Zhang J, Herren G, Müller T, Krattinger SG, Keller B, Jiang Y, González MY, Zhao Y, Habekuss A, Färber S, Ordon F, Lange M, Börner A, Graner A, Reif JC, Scholz U, Mascher M, Stein N (2019): Genebank genomics highlights the diversity of a global barley collection. Nature Genetics 51 (2):319–326. doi:10.1038/s41588-018-0266-x

[14] van Hintum TJL (1997): Central Crop Databases – an overview. Paper presented at the Central Crop Databases: Tools for Plant Genetic Resources Management,

[15] van Treuren R, van Hintum TJL (2014): Next-generation genebanking: plant genetic resources management and utilization in the sequencing era. Plant Genetic Resources 12 (3):298–307. doi:10.1017/S1479262114000082

[16] Wambugu PW, Ndjiondjop M-N, Henry RJ (2018): Role of genomics in promoting the utilization of plant genetic resources in genebanks. Briefings in Functional Genomics 17 (3):198–206. doi:10.1093/bfgp/ely014

[17] Weise S, Lohwasser U, Oppermann M (2020): Document or Lose It – On the Importance of Information Management for Genetic Resources Conservation in Genebanks. Plants 9 (8):1050. doi:10.3390/plants9081050

[18] Weise S, Oppermann M, Maggioni L, van Hintum T, Knüpffer H (2017): EURISCO: The European search catalogue for plant genetic resources. Nucleic Acids Research 45 (D1):D1003-D1008. doi:10.1093/nar/gkw755

[19] Wieczorek J, Bloom D, Guralnick R, Blum S, Döring M, Giovanni R, Robertson T, Vieglais D (2012): Darwin Core: An Evolving Community-Developed Biodiversity Data Standard. PLOS ONE 7 (1):e29715. doi:10.1371/journal.pone.0029715

DOI: 10.5507/vup.21.24459677.04

New insights on the genetic structure of lucerne with GBS markers

Marie Pégard[1], Philippe Barre[1] and Bernadette Julier[1]

[1] INRAE P3F, 86600 Lusignan, France

marie.pegard@inrae.fr

Abstract. GBS markers at the population level were used to study the genetic structure of lucerne in a set of 395 accessions from Europe, North and South America and China. More than 100K SNP without missing data were obtained. The structure revealed a certain genetic distance between European and American accessions, with a continuous variation between these groups. Contrastingly, Chinese accessions were genetically separated from the other accessions. This analysis is an invitation to go more deeply into a phenotypic analysis of these genetic resources to exploit them in breeding.

Keywords: alfalfa, breeding, genetic resources, *Medicago sativa* L., SNP

1 Introduction

Lucerne (*Medicago sativa* L.) originates from the Middle East but is grown worldwide. As a consequence, this species is adapted to various climatic conditions, from continental to arid climate. The dissemination history of the species has been described in historical documents, phenotypic and molecular studies [1]. Briefly, it has been introduced several times in Europe with invaders (Medes and Romans through northern Mediterranean Basin and Maures through southern Mediterranean Basin), and was introduced to the Americas during the European migrations from the end of 16th century. Lucerne moved to China about 2000 years ago [2]. The cultivated forms of the ssp. *sativa* have naturally hybridized with the wild and cold tolerant ssp. *falcata* that expanded in the Siberian region.

The genotyping by sequencing (GBS) method provides numerous markers at the population level and covers the alfalfa genome. [3]. They offer a means to study the structure of the genetic resources available in this species. The current breeding programmes, conducted in Europe, America or Asia, could be improved by using a larger genetic diversity. For this reason, the objective was to describe the genetic structure of a set of 395 lucerne accessions mostly coming from Europe, North and South America and China with GBS markers.

2 Material and Methods

In this study, we used 395 accessions comprised of 373 cultivars and 22 landraces whose autumn dormancy was mainly among 3 and 7 on a scale of 1 to 9 (1 slightly dormant and 9 very dormant). Their origin, based on the place they have been collected (landraces) or initially selected and registered (cultivars) was Europe (313 accessions), North America (45 accessions), South America (16 accessions), China (17 accessions), Middle East (3 accessions) and Japan (1 accession). The accessions were genotyped by GBS [4]. A subset of SNP without any missing value was sorted out to avoid an imputation step and only SNPs with a minor allelic frequency higher than 1% were kept, 109 K SNP were obtained. These genomic data were used to identify genetic clusters among these populations with the Discriminant Analysis of Principal Components (DAPC) method. The number of retained PC was fixed at 300, this number represented the number of PC that explained 90% of the variation. The genetic clusters were identified by using k-means, a clustering algorithm which found a given number (k) of groups by maximizing the variation between groups. The optimal number of clusters was the one that provided the lowest Bayesian Information Criterion (BIC), here seven. We then run a Principal Component Analysis (PCA). The accessions were grouped by using either the seven groups defined from DAPC analysis or an *a priori* classification into six groups based on the geographical origin of the accessions: "Europe", "North America", South America", "China", "Middle East", "Japan".

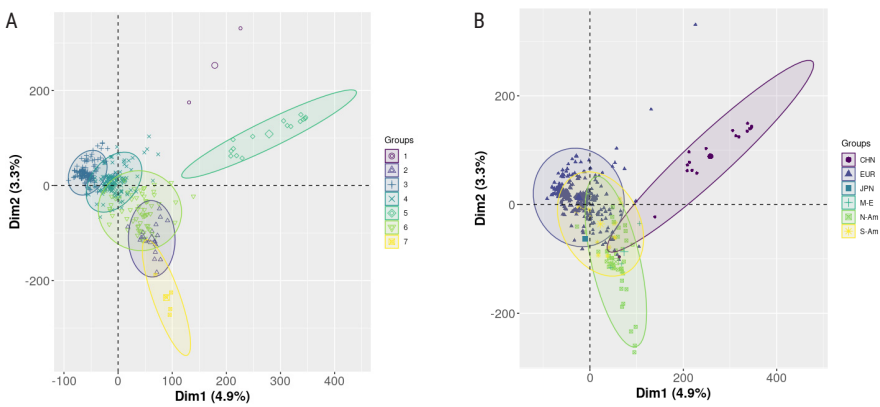


Fig. 1. Graphical representation of the first two dimensions of a principal components analysis for lucerne accessions. PC1 and PC2 explained 4.9% and 3.3% of total genetic variation. A: each group found with the DAPC procedure is in a different color. B: the individuals are colored according to their geographical origin; CHN: China, EUR: Europe, JPN: Japan, M-E: Middle-Est, N-Am: North America, S-Am: South-America.

3 Results

The Fig. 1 (A) represents the PCA with the accessions colored according to the seven DAPC groups. Two groups were clearly separated from the others: group 6 with 15 Chinese accessions and group 1 with two accessions only (a *falcata* type from Italy and a Hungarian variety). The five other groups showed a genetic continuum: group 3 with 139 accessions of European origin only (France and Northern Europe), group 7 with 151 accessions mostly of European origin (Southern and Eastern Europe), group 4 with 61 accessions of Europe, North and South America), group 5 with 21 USA and 1 Chinese accessions and group 2 containing 5 North-American accessions only. The European accessions as well as the American accessions were splitted into several groups. The group 4 probably illustrates the multiple origins of some varieties selected in the two continents.

When the clustering was based on the geographical origin of the accessions (Fig. 1 (B)), the Chinese accessions were again mostly separated from all other origins. The European and the North-American accessions had little overlapping, but South-American accessions interestingly overlapped these two groups. The three accessions from the Middle East were close to the American accessions and the Japanese accession was closer to the European-American groups than to the Chinese group.

The structure obtained in this study shows that breeding programmes conducted in Europe partly ignore genetic resources from North and South America even if there is a continuous variation among groups. Contrastingly, there is an almost complete separation among European-American accessions on one side and Chinese accessions on the other side. In this material, the autumn dormancy rating, when known, is not a relevant classification key (not shown). This analysis is an invitation to go more deeply into a phenotypic analysis of these genetic resources to exploit them in breeding. Further historical analysis of lucerne expansion in the world could be also interesting.

4 Acknowledgements

This study was supported by EUCLEG project (UE, H2020 n°727312). We thank Sabrina Delaunay and Sébastien Blugeon for their contribution to genotyping and the Centre of Biologic Resources of INRAE (URP3F). We also thank all the institutes and breeding companies that accepted to include their varieties in this study.

References

[1] Prosperi, J.M., Muller, M.H., Ronfort, J., Fourtier, S., Sampoux, J.P., Jenczewski, E. [2014]: Alfalfa domestication history, genetic diversity and genetic resources. Legume Perspectives 4, 13–14.

[2] Basigalup, D., Irwin, J., Mi, F., Abdelguerfi-Laouar, M (2014).: Perspectives of alfalfa in Australia, China, Africa and Latin America. Legume Perspectives 4, 9–10.
[3] Julier, B., Barre, P., Lambroni, P., Delaunay, S., Thomasset, M., Lafaillette, F., Gensollen, V. (2018): Use of GBS markers to distinguish among lucerne varieties, with comparison to morphological traits. Mol. Breed. 38, 133.
[4] Julier, B., Blugeon, S., Delaunay, S., Mappa, G., Ruttink, T., Pégard, M., Barre, P. (2021): Optimisation of GBS protocols for efficient genotyping of forage species. Proceedings of Eucarpia Forage Crops and Amenity Grasses section conference, Freising, Germany.

DOI: 10.5507/vup.21.24459677.05

Phenotyping Drought Tolerance and Root Morphology in Perennial Ryegrass

Silvia Bachmann-Pfabe[1], Stephan Hartmann[2], Peter Westermeier[2] and Evelin Willner[1]

[1] Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gene Bank, Satellite Collections North, Inselstrasse 9, 23999 Malchow/Poel, Germany
[2] Bavarian State Research Center for Agriculture (LfL), Institute for Crop Science and Plant Breeding, Am Gereuth 4, 85354 Freising, Germany

pfabe@ipk-gatersleben.de

Abstract. Diploid perennial ryegrass progenies derived from a cross of parent plants differing in drought tolerance were phenotyped in rainout shelters for two years at two locations. Four crossing populations with considerable drought tolerance were identified, indicated by a high number of vigorous plants (80 - 90 %) after two drought periods per year. Preliminary results of experiments in mini-rhizotrons identified the total root length as one component of drought tolerance in perennial ryegrass which should be studied in more detail.

Keywords: rainout shelter, vigour, grasses, genetic resources.

1 Introduction

Perennial ryegrass (*Lolium perenne* L.) is one of the most important forage and turf grass species in temperate regions worldwide, but is generally sensitive to heat and summer drought due to its shallow root system [1,2]. We aimed to identify *L. perenne* populations tolerant to periodical drought and to understand inheritance patterns and drought tolerance mechanisms by crossing drought tolerant and susceptible parents and studying their root morphology.

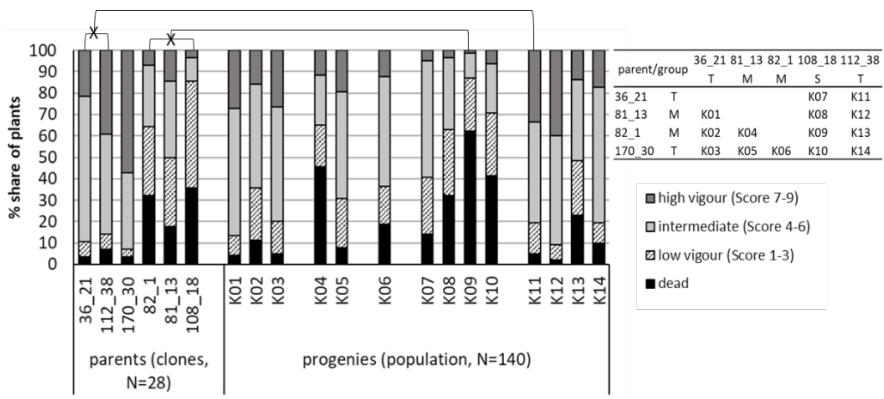
2 Material and Methods

14 biparental crossing populations (K01 – K014) were cultivated in rainout shelter experiments in the years 2017 and 2018 at two sites (Malchow/Poel, Freising/Pulling, Germany). Each crossing population comprised 140 individual genotypes resulting from the cross of drought tolerant (T), medium tolerant (M) and susceptible (S) parental plants identified in a previous experiment (Fig.1). Each

genotype/progeny was cloned 3 times. One clone each was planted in the rainout shelter in Poel and Freising. The remaining clones were distributed among the sites so that each population was represented by half of the individuals (70) in the field. Progenies were compared to their cloned parental plants in an augmented randomised block design. Drought periods were applied in early spring and in summer by keeping the soil moisture below the permanent wilting point for six weeks. Plant vigour was scored on a scale from 0 (dead), 1 (low) to 9 (high). For each population the number of plants within the following classes was counted: dead (score 0), "low vigour" (score 1–3), "intermediate vigour" (score 4–6) and "high vigour" (score 7–9). The dry matter yield (DM) of individual plants was determined after cutting, oven drying at 60 °C and weighing. Two IPK Gene Bank accessions serving as the source of a very tolerant (112_38 from GR5559) and a medium tolerant parent (82_1 from GR3109), were studied for their root morphology. Ten pre-germinated seeds per accession were transplanted into rhizotrons of 20 x 20 cm size, filled with a turf-based planting substrate. Roots were separated from the turf substrate by a synthetic membrane with 20 µm mesh size. Seedlings were watered according to their needs and cultivated for 3 weeks at 15/10 °C (day/night) and 12 hours of light. At harvest, roots were scanned at 1200 dpi (HP Scanjet 4890) and images were analysed with saRIA software [3]. One-way analysis of variance (package lme4, R Studio) was used to test for significant differences between the two accessions.

3 Results and Discussion

At Malchow/Poel, most of the crossing populations revealed a high share of very vigorous genotypes at the end of 2017, indicating that *L. perenne* can withstand single drought events (data not shown). After recurrent drought events in 2018, differences within and between populations became more pronounced (Fig. 1).



parent/group		36_21 T	81_13 M	82_1 M	108_18 S	112_38 T
36_21	T				K07	K11
81_13	M	K01			K08	K12
82_1	M	K02	K04		K09	K13
170_30	T	K03	K05	K06	K10	K14

Fig. 1. Vigour of the parental plants and the crossing populations at the end of 2018 after two years with two drought stress periods per year at the Malchow/Poel experimental site. Marked are one of the best and the worst performing population and their respective parents.

In worst performing crossing populations (K04, K08, K09, K10), the biomass scoring in November 2018 revealed a high share of dead plants (32–62 %), but a low share of genotypes (13–37 %) with intermediate to high vigour. In contrast, the best crossing populations (K01, K03, K11, K12, K14) still exhibited a high number of medium to very vigorous genotypes (80–90 %). These observations coincided with the harvested DM and the plant vigour scores recorded at Freising. Comparisons of the DM yield from the field and rainout shelter in Malchow identified K11 as the population which ranked best in the field and was amongst the highest yielding populations after drought stress in the shelter. It resulted from the cross of the tolerant parents 36_21 and 112_38. Parent 112_38 is a selection of the accession GR5559 originating from the Satu Mare region in Romania, while parent 36_21 was selected from a breeding line. In contrast, parent 82_1, a selection from accession GR3109 (cultivar Pippin, Denmark), generally produced less tolerant progenies (Fig. 1). *First results from the rhizotron experiments indicate that g*enotypes of accession GR5559 exhibit a faster and stronger root development at an early stage of plant growth than those of GR3109 (Tab. 1). However, differences between both populations were not statistically significant. Nevertheless, total root length tended to differentiate most between the two accessions ($p \leq 0.066$).

Table 1. Selected root morphology traits of two *L. perenne* accessions after cultivation of 10 genotypes per accession in mini-rhizotrons for three weeks.

Trait	Accession GR3109			Accession GR5559		
	Mean	SD	CI	Mean	SD	CI
TArea [mm²]	480	204	328–631	644	225	500–788
TLength [mm]	1623	676	1052–2194	2356	916	1814–2898
TVolume [mm³]	122	55.6	71.9–172	161	82.4	113–208
SA [mm²]	1571	678	1033–2109	2131	835	1620–2642
SRL [mm/mm³]	14.1	2.87	10.4–17.8	17.1	6.76	13.5–20.6

GR3109 source of the medium tolerant parent 82_1, GR5559 source of drought tolerant parent 112_38, CI = confidence interval at confidence level 0.95; TArea = total root area, TLength = total root length, TVolume = total root volume, SA = total surface area, SRL = specific root length.

Progenies with a high drought tolerance were multiplied and included as new accessions GR13409-GR13418 into the IPK Gene Bank. Root morphology of the specific parent plants will be further studied with special emphasis on total root length.

Acknowledgements

The research leading to these results received funding from the German Federal Ministry of Food and Agriculture (BLE Innovationsförderung; FKZ: 2818209615, DRYeGRASS).

References

[1] Hatier, J.H.B., Faville, M.J., Hickery, M.J., Koolaard, J.P., Schmidt, J., Carey, B.L., Jones, C.S.: Plant vigour at establishment and following defoliation are both associated with responses to drought in perennial ryegrass. J. Exp. Bot. 65, 5823–5834 (2014).

[2] Wedderburn, M.E., Crush, J.R., Pengelly, W.J., Walcroft, J.L. (2010): Root growth patterns of perennial ryegrasses under well-watered and drought conditions. New Zeal. J. Agr. Res. 53, 377–388.

[3] Narisetti, N., Henke, M., Seiler, C., Shi, R., Junker, A., Altmann, T., Gladilin, E. (2019): Semi-automated Root Image Analysis (saRIA). Sci. Rep. 9, 19674.

DOI: 10.5507/vup.21.24459677.06

Genomic and phenotypic diversity for adaption to future climate in natural populations of perennial ryegrass

José Luis Blanco-Pastor[1], Thomas Keep[1], Philippe Barre[1], Abraham Escobar-Gutiérrez[1], Evelin Willner[2], Klaus J. Dehmer[2], Matthew Hegarty[3], Hilde Muylle[4], Tom Ruttink[4], Isabel Roldán-Ruiz[4], Stéphanie Manel[5], Florence Volaire[6] and Jean-Paul Sampoux[1]

[1] INRAE, URP3F, Centre Nouvelle-Aquitaine-Poitiers, Lusignan, France
[2] Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Malchow/Poel, Germany
[3] Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth University, Aberystwyth, UK
[4] Flanders Research Institute for Agriculture, Fisheries and Food (ILVO) - Plant Sciences Unit, Melle, Belgium
[5] CEFE, University of Montpellier, CNRS, EPHE-PSL University, IRD, Montpellier, France
[6] CEFE, University of Montpellier, INRAE, CNRS, EPHE, IRD, Montpellier, France

jose.luis-blanco.pastor@inrae.fr

Abstract. Germplasm from perennial ryegrass (*Lolium perenne* L.) natural populations is useful for breeding because of its adaptation to a wide range of climates. We identified adaptive loci in perennial ryegrass and their association with seasonal growth traits and climatic gradients. Adaptive loci associated with autumn growth under cold winters and summer growth under dry and long summers were common to only a limited extent, pointing to a moderate trade-off along the winter-summer stress gradient due to antagonistic pleiotropy. Meanwhile, most adaptive loci were found specific to one type of stress or the other. It should therefore be possible to combine adaptation to winter and summer stresses by genetic recombination. This would be particularly useful in view of foreseen increasing climate variability due to climate change in Europe. On the other hand, the climatic distribution models of adaptive allele frequencies revealed a risk of local extinction in regions with much drier and warmer summers in the future such as the Iberian Peninsula and southern France.

Keywords: genetic resources, grasslands, adaptation, climate change, perennial ryegrass.

1 Introduction

Natural diversity of perennial ryegrass is useful for breeding as it includes adaptation to a very wide range of environmental conditions, but such diversity is locally threatened by changing climate. Perennial ryegrass will likely require shifts in allele frequencies at adaptive loci to adapt to new climatic constraints. This study aimed to identify a set of adaptive loci involved in seasonal growth rate in perennial ryegrass, explored the trade-offs between long-dry summer conditions and cold winter conditions in this species and investigate whether the intra-population adaptive genetic diversity present is sufficient to allow for adaptation to climate change.

2 Material and Methods

To identify a set of adaptive loci involved in oligogenic and polygenic adaptations in *Lolium perenne* (perennial ryegrass) we used the CANCOR test, a recently proposed multivariate method based on canonical correlations [1]. The CANCOR test was applied to data collected on 385 natural populations of perennial ryegrass. Then we estimated most suitable allele frequencies in the future climate using linear models of environmental distribution of allele frequencies. For each adaptive SNP, a logistic regression between the alternative allele frequencies and the selected bioclimatic descriptors was implemented using the "mutinom" R function with a stepwise process. Finally, we estimated genetic vulnerability of populations as the euclidean distance between observed allele frequencies in genotyped populations and most suitable allele frequencies in the future climate.

3 Results and Discussion

We detected polygenic adaptations to low winter temperatures, *i.e.* 370 outlier SNP loci associated with frost tolerance and 169 SNP loci associated with spring growth under cold winter conditions. On the other hand, variants of five SNP loci were found associated with late heading date and low winter temperatures, indicating an oligogenic adaptation to cold winters. The CANCOR test also detected polygenic adaptations to long and dry summer environments, *i.e.* 61 SNP loci associated with high investment in sexual reproduction and 33 SNP loci associated with higher summer growth rate of canopy biomass under heat stress (which developed mainly as reproductive tillers). Two outlier SNP loci variants were also found associated with high lignin content of the vegetative biomass and long summer environments. They likely report for an oligogenic adaptation to such environments as high lignin content corresponds to a high density of leaf tissues known to contribute to tolerance to drought stress [2].

The CANCOR test revealed that loci associated with adaptation to cold winters and dry and long summers were common to only a limited extent, pointing to a moderate trade-off along the winter-summer stress gradient due to antagonistic pleiotropy (Fig. 1). Most adaptive loci were found specific to either summer or winter stress. Although difficult, given the polygenic nature of adaptive traits, it should be possible to combine adaptation to winter and summer stresses to some extent by genetic recombination. This would be particularly useful in view of foreseen increasing climate variability due to climate change in Europe.

Allele distribution model projections identified the risk of maladaptation (genetic off-set) of local natural populations across Europe (Fig. 2). These projections predicted a genetic off-set in regions with much drier and warmer summers in the future such as the Iberian Peninsula and southern France.

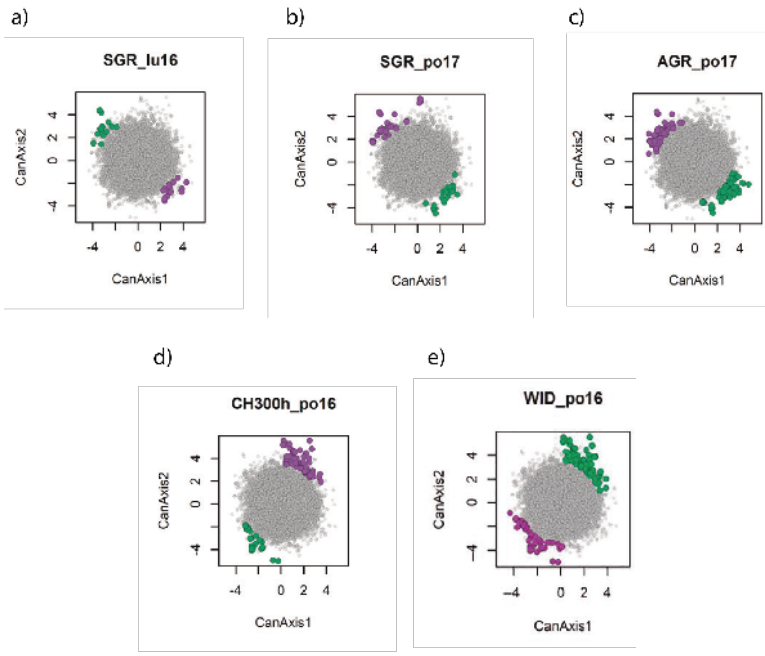


Fig. 1. Examples of outlier SNP loci of perennial ryegrass revealed by the CANCOR test. a) Summer growth rate under long summer climate, b) summer growth rate under cold winter climate, c) autumn growth rate under cold winter climate, d) canopy height 300 degrees days before heading (indicator of spring growth rate) under cold winter climate and (e) frost damage under cold winter climate. It can be noticed that a set of outlier loci are associated with both (a) and (b, c) but with opposite alleles (antagonistic pleiotropy). On the other hand, different sets of loci are associated with (a, b, c) and (d, e), allowing for a combination of high summer growth rate and high spring growth rate (and less winter damage) under cold winter conditions (from [1]). Purple and green colours indicate positive and negative correlations, respectively, between the locus alternative allele frequency and the phenotypic variable.

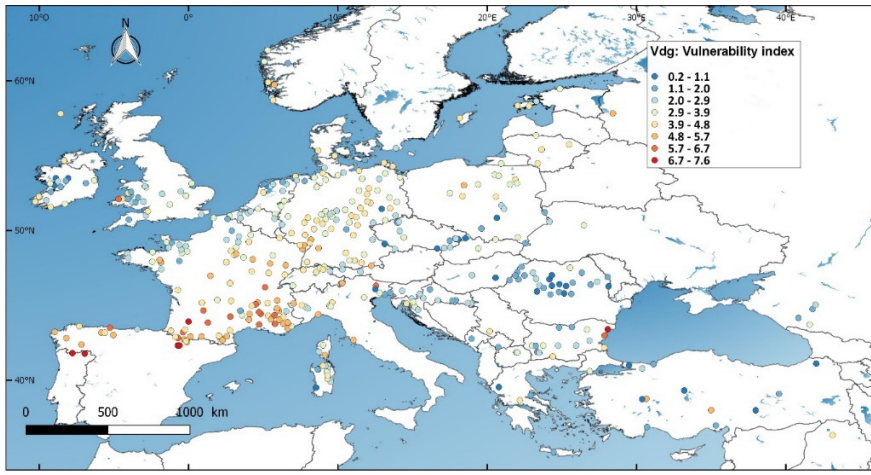


Fig. 2. Vulnerability of local natural populations of perennial ryegrass to the predicted climate for the period 2041–2071 (RCP8.5, RCA4) estimated from 633 adaptive loci (increasing vulnerability from cold to warm colours).

References

[1] Blanco-Pastor, J.L., Barre, P., Keep, T., Ledauphin, T., Escobar-Gutiérrez, A., Roschanski, A.M. et al. (2021): Canonical correlations reveal adaptive loci and phenotypic responses to climate in perennial ryegrass. Mol. Ecol. Res. 21, 849–870.

[2] Wilson, P. J., Thompson, K. E. N., and Hodgson, J. G. (1999): Specific leaf area and leaf dry matter content as alternative predictors of plant strategies. New Phytol. 143, 155–162.

DOI: 10.5507/vup.21.24459677.07

Variability for organic matter digestibility at heading date in a collection of perennial ryegrass genotypes

Vincent Colas[1], Philippe Barre[1], Frederik van Parijs[2], Lukas Wolters[3], Yannik Quitté[4], Isabel Roldán-Ruiz[2] and Hilde Muylle[2]

[1] INRAE, UR P3F, RD 150 Le Chêne, 86600, Lusignan, France
[2] ILVO, Plant Sciences Unit, Caritasstraat 39, 9090 Melle, Belgium
[3] DSV zaden Nederland B.V., Zelder 1, 6599 EG Ven Zelderheide, The Netherlands
[4] DSV France, Lieu-dit La Planche 49350 Les Rosiers sur Loire, France

hilde.muylle@ilvo.vlaanderen.be

Abstract. Digestibility is essential to determine the value of a forage perennial ryegrass variety. We observed a large variability for Organic Matter Digestibility (OMD) in a panel of 580 perennial ryegrass genotypes when harvested at heading date. This variability was explained by the proportion of blades and by both, the cell wall content (NDF content) and its digestibility (NDFD). These last two traits showed no correlation in this experiment. The broad sense heritabilities of OMD, NDF content and NDFD were 0.77, 0.76 and 0.73, respectively. Blades showed slightly higher OMD than 'stems' (sheaths and flowering stems) but the distributions overlapped largely with some 'stems' showing higher OMD than some blades. These findings for the whole collection were also obtained within maturity groups and are encouraging for breeding for high OMD *via* a decrease of NDF content and an increase of NDFD in both blades and 'stems'.

Keywords: digestibility, cell wall content, *Lolium perenne* (L.).

1 Introduction

Digestibility of organic matter (OMD) is a major selection criterion in forage grass species breeding [1] [2]. Indeed, OMD influences fodder intake, milk production and meat production. For example, an increase of 1% of OMD generally leads to a 3.2% increase in average daily gains of beef cattle [3]. Organic matter consists of water soluble components which are almost completely digestible, and cell walls (NDF content) which are partially digestible. Increase in OMD could be obtained by decreasing NDF and/or by increasing the cell wall digestibility (NDFD).

The objective of this study was to survey the variability of OMD in a large set of perennial ryegrass genotypes. As OMD varies throughout the season according to the developmental stage of the ryegrass plant, we monitored closely the development of each plant and harvested plant materials for biochemical analysis when each particular plant had reached the 'heading stage'. The origin of this variability was explored: NDF content *versus* NDFD and blades *versus* 'stems' (sheaths and flowering stems).

2 Material and Methods

A set of 580 perennial ryegrass genotypes (209 from natural populations) [4] was cultivated outdoors in pots (12 L) at Melle in Belgium starting at the beginning of March 2012. The experiment was set-up as a randomized block design with three replicates. At its respective heading date, each plant was harvested and the plant material was split in two fractions: blades and 'stems' (sheaths and flowering stems). Each part was dried for 48 h at 70°C and grinded. NIRS was used to determine the organic matter content (OM), the organic matter digestibility (OMD) and the NDF cell-wall content [5]. Digestibility of NDF (NDFD) was calculated as 100 - 100 x (100 - OMD)/NDF [6] [7].

3 Results and Discussion

As expected, given the large genetic diversity present in perennial ryegrass, we observed a large phenotypic variability for quality traits (Table 1). The results are consistent with the findings of previous studies in which the high nutritive value of perennial ryegrass in comparison to other forage grasses was demonstrated, but showing also the existence of useful variation [1] [2] [8] [9]. The variability of OMD was partly explained by heading date (not shown). In particular, a group of early plants (before May 26 or 900 GDD) showed a higher OMD (88% OM) than the rest of the genotypes (86% OM).

Table 1. Variability of traits. Std: standard deviation. GDD: growing degree days with 0°C as base temperature and starting March 1.

Traits	min	max	mean	std
Heading date (GDD)	700	1402	982	159
Blade proportion (% w/w)	23	93	47	9
OMD (% OM)	79	92	87	2
NDF (% DM)	35	58	45	3
NDFD (% NDF)	61	80	70	2

The variability of OMD was partly linearly explained by the proportion of blades (R^2=0.32) because i/ blades showed a slightly higher OMD than 'stems' (87% OMD *vs* 86% OMD) and ii/ OMD increased with blade proportion in

both blades (R^2=0.19) and 'stems' (R^2=0.20). The higher digestibility of blades compared to 'stems' is generally observed in grasses [8]. In our case, this higher OMD of blades compared to 'stems' was due to a lower NDF content (42% DM *vs* 48% DM) but not to a higher NDFD (70% NDF *vs* 71% NDF) in blades compared to 'stems'. Nevertheless, we observed that the distribution of OMD in blades overlapped the one in 'stems' keeping in mind that the 'stems' were composed of sheaths and flowering stems which were not mature. A large variability of OMD remained within blades and 'stems', which could be explained by both NDF content and NDFD (Fig. 1). These findings for the whole collection were also obtained within maturity groups (not shown). It is worthy to note that there was no correlation between NDF content and NDFD in both blades and 'stems' (not shown). Finally, a large part of the variability of OMD, NDF and NDFD could be explained by genetic variability with broad sense heritabilities of 0.77, 0.76 and 0.73, respectively.

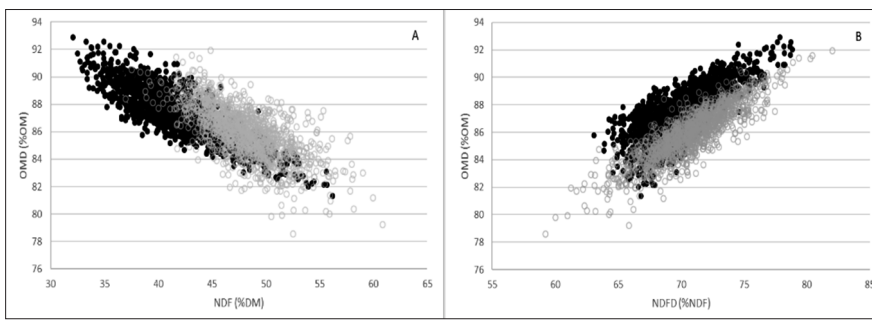


Fig. 1. Relationships between the digestibility of organic matter (OMD) and (A) the NDF content and (B) the NDFD in blades (black) and in 'stems' (grey).

References

[1] Sampoux, J.-P. et al (2011): Breeding perennial grasses for forage usage: An experimental assessment of trait changes in diploid perennial ryegrass (Lolium perenne L.) cultivars released in the last four decades. Field Crops Res. 123:117–129.

[2] Wilkins, P. (1997): Useful variation in in vitro digestibility within perennial ryegrass. Euphytica 93:249–255.

[3] Casler, M., Vogel, K. (1999): Accomplishments and impact from breeding for increased forage nutritional value. Crop Sci. 39:12–20.

[4] Veeckman, E. et al (2019): Overcoming challenges in variant calling: exploring sequence diversity in candidate genes for plant development in perennial ryegrass (Lolium perenne). DNA Res. 26:1–12.

[5] Muylle, H., Van Waes, C., Van Parijs, F., Obianugba, G., Baert, J. et al. (2012): Genetic diversity for cell wall digestibility in a diverse Lolium perenne collection. In: Breeding Strategies for sustainable forage and turf grass improvement, Springer, Dublin, pp. 261–267.

[6] Struik, P.C. (1983): Physiology of forage maize (Zea mays L.) in relation to its production and quality. Landbouwhogeschool Wageningen (1983).
[7] van Parijs, F., Van Waes, C., Vandecasteele, B., Haesaert, G., Roldan-Ruiz, I., Muylle, H. (2018): The optimal lignin quantification method to breed for an improved cell wall digestibility in perennial ryegrass. Grass Forage Sci. 73:101–111.
[8] Terry, R.A., Tilley, M.A. (1964): The digestibility of the leaves and stems of perennial ryegrass, cocksfoot, timothy, tall fescue, lucerne and sainfoin, as measured by an in vitro procedure. Grass Forage Sci. 19:363–372.
[9] Buxton, D.R., Casler, M.D. (1993): Environmental and genetic effects on cell wall composition and digestibility. In: Forage cell wall structure and disgestibility, ASA-CSSA-SSSA, Madison, pp. 685–714.

DOI: 10.5507/vup.21.24459677.08

Red clover adaptation to a Nordic climate

Stefano Zanotto[1], Helga Amdahl[2] and Åshild Ergon[1]

[1] Faculty of Biosciences, Dept. of Plant Sciences, Norwegian University of Life Sciences, Ås, Norway
[2] Graminor AS, Ridabu, Norway

stefano.zanotto@nmbu.no

Abstract. We analyzed traits related to yield and persistency from a single field trial over 2 years in south-eastern Norway involving 110 red clover (*Trifolium pratense* L.) populations of various origin. A significant differentiation was found between Nordic populations and other material. The first group showed better stand density and yield over time, while the latter tended to grow longer during the first autumn after establishment. Positive correlations of stand density and yield with resistance to clover rot in the field and freezing tolerance measured under controlled conditions suggest that these traits are important to improve persistency in the south-eastern.

Keywords: freezing tolerance, persistency, yield.

1 Introduction

High yield and persistency are the main breeding goals for red clover *(Trifolium pratense L.)* in the Nordic countries [1]. The major cause of poor persistency at northern latitudes is poor winter survival, which is a complex trait affected by many different stress factors [2]. Furthermore, the climate change will result in higher temperature and less stable winters at those locations, resulting often in the lack of stable snow cover and sudden frost events. In this scenario the breeding of populations with a wide genetic base for traits related to biotic and abiotic stress resistance may be a good strategy for obtaining persistent and high yielding populations over time [3].

2 Material and Methods

A total of 110 red clover populations were sown in the summer of 2018 at the Graminor AS field station Arneberg, Norway, (61°22'N, 11°20'E), in plots of 4x1.25 m, in a row column design with two complete replicated blocks. The populations were a mix of cultivars and landraces mainly from Nordic and central Europe. Twelve phenotypic traits were recorded at plot level from autumn 2018 to autumn 2020.

The traits were: Juvenile plant density 2018 (JPD), stand height in the autumn 2018 and spring 2019 (SH1, SH2), flowering date 2019 (FL), dry matter yield 2019 and 2020 (DM1, DM2), protein content (PT), stand density in the autumn and spring of 2019 (SD1, SD2) and 2020 (SD3, SD4), clover rot resistance in spring 2019 (SC). In addition, an experiment under controlled conditions was conducted for the determination of freezing tolerance as temperature causing 50% of plant death (Lt50) as described by Zanotto et al. [3].

Variance component analyses of the field data were conducted using the residual maximum likelihood (REML) procedure with the remlf90 function [4]. A random effect capturing spatial heterogeneity at plot level within the trial was fitted using a bi-splines surface covering row and column axes, nested within the two replicated blocks. Principal component analysis and phenotypic correlation analysis were conducted on averaged data. All the statistical analyses were done in RStudio version 1.1.463 [4].

3 Results and Discussion

Most of the variation is captured by PC1 (57.9 %) while PC2 accounts for a significantly smaller part (9.7 %) (Fig. 1). PC1 separated Nordic populations from the rest. The Nordic material was characterized by later flowering, better stand density in spring and autumn, higher DM yield and protein content, less clover rot and better freezing tolerance, while material from central and western Europe and the USA had taller stands in late autumn after establishment.

Table 1. Phenotypic correlation coefficients and entry-mean broad sense heritability (h^2_b) estimates ($p < 0.05$) for the traits recorded in the period from autumn 2018 to autumn 2020 in plot trials with 110 red clover populations at Arneberg, Norway. Freezing tolerance from a controlled conditions experiment is also included in the correlation analysis. See material and methods section for a description of the traits.

Trait	JPD	SH1	SH2	SD1	SC	FL	SD2	DM1	PT	SD3	SD4	DM2
SH1	NS*											
SH2	NS	-0.56										
SD1	NS	-0.61	0.82									
SC	NS	-0.58	0.60	0.75								
FL	NS	-0.67	0.47	0.46	0.44							
SD2	0.26	-0.57	0.69	0.80	0.56	0.50						
DM1	NS	-0.30	0.59	0.71	0.53	0.22	0.65					
PT	NS	-0.68	0.51	0.55	0.57	0.59	0.59	0.38				
SD3	NS	-0.76	0.63	0.75	0.70	0.61	0.71	0.43	0.67			
SD4	NS	-0.80	0.70	0.78	0.67	0.65	0.79	0.47	0.71	0.91		
DM2	NS	-0.77	0.66	0.73	0.67	0.62	0.75	0.45	0.69	0.96	0.95	
Lt50	NS	0.34	-0.20	-0.25	-0.20	-0.19	-0.22	NS	NS	-0.23	-0.23	-0.24
h^2_b	0.80	0.89	0.60	0.86	0.65	0.67	0.79	0.63	0.73	0.92	0.91	0.93

*not significant

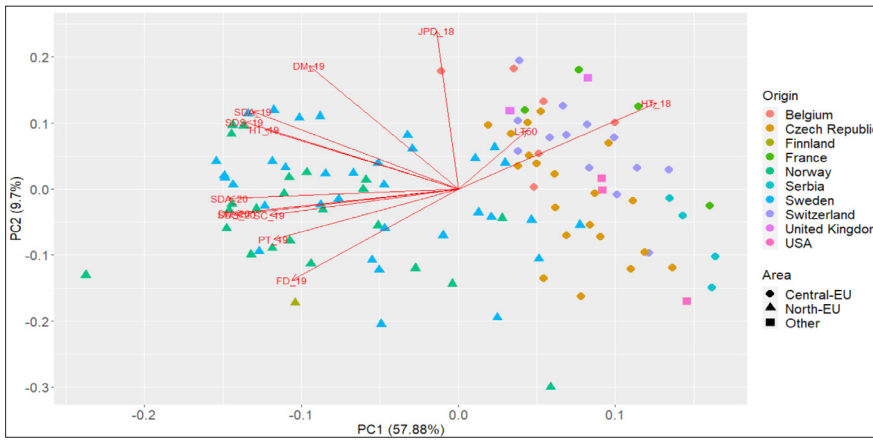


Fig. 1. Principal component analysis on averaged data for the field traits and LT50.

However, the Nordic populations had larger stand height in the spring. All the field traits but juvenile plant density in the autumn were significantly correlated to each other (Table 1). Freezing tolerance was negatively correlated (lower Lt50 means better freezing tolerance) with all field traits but juvenile plant density, DM yield and protein content. All the field traits had significant genotypic variance components and had relatively high to high heritability (0.60–0.93), which is promising for further improvement of Nordic material through breeding. The visible separation between the Nordic material and the rest and the strong correlations between DM yield and stand density in both growing seasons clearly shows the importance of breeding material adapted to specific environments. Nordic material had better freezing tolerance and clover rot resistance, which resulted in higher yield in the subsequent growing season. The taller stands of the non-Nordic material in the autumn after establishment is probably due to a tendency of these populations to continue growing longer in the late summer/autumn, while the Nordic material tended to stop their growth earlier, possibly prioritizing accumulation of reserves in the overwintering parts of the plants. Interestingly, stand density and yield in 2020 had higher heritabilities and higher correlations to freezing tolerance and resistance to clover rot than in 2019. This can be interpreted as an accumulating effect of winter stresses over time. It is worth to consider that this study presented data for only one location, and lower heritability values can be expected from multi-environment field trials, especially for traits characterized by high GxE.

Acknowledgements

This research was part of EUCLEG (EU Project No. 727312), and we acknowledge the overall planning of field experiments by WP, species and project leader.

References

[1] Helgadóttir, Á., Larsen, A., Marum, P., Fritsen, H., Lindvall, E., Miettinen, E. (2002): Prebreeding of red clover (Trifolium pratense L.) for northern areas. Acta Agric. Scand., Sect. B - Soil & Plant Sci. 50, 187–190.

[2] Bélanger, G., Castonguay, Y., Bertrand, A., Dhont, C., Rochette, P. et al. (2006): Winter damage to perennial forage crops in eastern Canada: causes, mitigation, and prediction. Can. J. Plant Sci. 86, 33–47 (2006).

[3] Zanotto, S., Palmè, A., Helgadóttir, À., Daugstad, K., Isolahti, M. et al. (2021): Trait characterization of genetic resources reveals useful variation for the improvement of cultivated Nordic red clover. J. Agron. Crop Sci. 207, 492–503.

[4] R Core Team: R (2018): A Language and Environment for Statistical Computing. R Core Team.

DOI: 10.5507/vup.21.24459677.09

Phosphorus utilisation capacity of forage legumes from recycling products

Yue Hu[1,2], Klaus J. Dehmer[1], Evelin Willner[1],
Silvia Bachmann-Pfabe[1] and Bettina Eichler-Löbermann[2]

[1] Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gene Bank, Satellite Collections North, Inselstrasse 9, 23999 Malchow/Poel, Germany
[2] University of Rostock, Agronomy and Crop Science, Justus-von-Liebig Weg 6, 18059 Rostock, Germany

hu@ipk-gatersleben.de

Abstract. Alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.) are important forage legumes cultivated around the world. Phosphorus (P) plays an important role in their growth. However, the interspecific and intraspecific P efficiency of alfalfa and red clover to utilise P from different fertilisers are less well studied. A field study was conducted at the University of Rostock, North-Eastern Germany, to test the efficiency of selected accessions of alfalfa and red clover in utilising P from different sources, where eight accessions of each species were selected for diverse variation of geographic origin, sample status and plant P content. Six treatments (no P, triple superphosphate, biomass ash, manure, biowaste compost and biowaste compost+triple superphosphate) were compared in a randomised split-plot design. Significant interspecific and intraspecific differences in plant P uptake were found in studied accessions of both species, where red clover had a significantly higher average P uptake than alfalfa ($P < 0.05$). P recycling products significantly increased P uptake in both species ($P < 0.05$), which follows the order biowaste compost > manure > biomass ash.

Keywords: P efficiency, P fertilisers, P recycling products, alfalfa, red clover.

1 Introduction

Phosphorus (P) plays an important role in agricultural production systems. Many studies have shown that P recycling products have the potential to improve soil P availability and crop growth. Alfalfa (*Medicago sativa L.*) and red clover (*Trifolium pratense* L.) are both herbaceous perennial legumes and widely cultivated as forage crops. Several studies regarding the effects of manure and sewage sludge on alfalfa and red clover showed a general positive effect in respect of improving plant growth and nutrient level. However, potential differences in the intraspecific efficiency of various accessions of alfalfa and red clover in utilis-

ing P from soil and different fertilisers are unknown and deserve more research in the future. The objectives of this study are to 1) examine the P efficiency of different accessions of alfalfa and red clover, 2) explain the differences in interspecific and intraspecific P efficiency and 3) assess the ability of alfalfa and red clover to utilise P from recycling products.

2 Material and Methods

In a preliminary experiment in 2019, 147 and 120 accessions of alfalfa and red clover, respectively, were cultivated for the selection of accessions suitable for this research. Eight accessions of alfalfa and red clover were then selected based on parameters including geographic origin of the plant material, sample status, plant P content, maturity group, etc. (Table 1). More information of studied accessions is available in homepage of National Inventory on Plant Genetic Resources in Germany [1].

Table 1. Geographic origin, sample status (SAMPSTAT) and plant P content of selected accessions of alfalfa and red clover.

Alfalfa				Red clover			
Accession	Origin	SAMPSTAT	Plant P content (mg 100 g^{-1} dry mass)	Accession	Origin	SAMPSTAT	Plant P content (mg 100 g^{-1} dry mass)
LE2812	YEM	300	428.84	LE1731	KGZ	300	394.64
LE2368	FRA	500	412.62	LE1423	FIN	400	366.68
LE2370	DNK	500	394.25	LE1391	GBR	200	355.96
LE2521	DEU	500	379.55	LE2750	HRV	100	343.30
LE713	ROU	500	303.28	LE1599	DEU	300	316.92
LE888	DEU	500	290.77	LE1775	RUS	100	297.70
LE2669	ROU	300	251.48	LE1804	SUN	999	283.21
LE2511	FRA	500	243.82	LE1937	DEU	100	272.14

Origin: Country codes according to ISO 3166; SAMPSTAT:100 = wild; 200 = weedy; 300 = traditional cultivar / landrace; 400 = breeding / research material; 500 = advanced or improved cultivar; 999: other).

A field trial was conducted on a long-term experiment field at Rostock University. The complete field trial was arranged in a randomized split-plot design with four blocks as replications. Each block consisted of three large plots, supplied with organic fertilisers. Within each large plot, inorganic fertilisers were further applied in three subplots. This combination of organic and inorganic fertilisers resulted in a total of nine treatments: control (no P), triple superphosphate (TSP), biomass ash (ash), cattle manure (manure), biowaste compost (compost), and the combined treatments compost+TSP, manure+ash, manure+TSP and compost+ash. In this study, only the first six treatments were studied. Seeds of eight accessions of alfalfa and red clover, respectively, were sown in April 2020. Aboveground biomass was harvested in July and September.

Table 2. Plant P uptake of selected accessions of alfalfa and red clover of the six studied treatments (no P, TSP, ash, manure, compost and compost+TSP) with ± standard deviation.

P uptake (kg ha^{-1})		Treatment						
Species	Accession	No P	TSP	Ash	Manure	Compost	Compost+TSP	Average
Alfalfa	LE 2812	7.49±1.12^{Ac}	9.05±1.51^{Ab}	9.88±1.59^{Aa}	10.63±2.48^{Aa}	9.86±0.96^{Ab}	11±1.48^{Ab}	9.65±1.84^{c}
	LE 2368	8.9±1.41^{BCbc}	11.67±2.39^{BCab}	13.17±1.84^{ABa}	11.89±2.03^{BCa}	15.08±2.35^{ABa}	16.59±0.95^{Aa}	12.88±3.04^{ab}
	LE 2370	13.32±2.67^{Aa}	12.06±1.41^{Aa}	12.25±1.41^{Aa}	13.52±0.76^{Aa}	13.31±1.71^{Aa}	15.2±2.4^{Aa}	13.27±1.9^{a}
	LE 2521	11.43±1.81^{BCab}	11.89±0.34^{ABCab}	10.31±1.58^{Ca}	12.51±1.39^{ABCa}	14.59±2.27^{ABa}	15.26±1.24^{Aa}	12.62±2.25^{ab}
	LE 713	9.34±1.97^{Bbc}	11.02±1.3^{ABab}	11.15±1.09^{ABa}	12.46±3.8^{ABa}	14.51±3.18^{ABa}	16±2.08^{Aa}	12.41±3.15^{ab}
	LE 888	10.21±1.49^{Cabc}	11.54±2.14^{Cab}	11.91±0.7^{BCa}	12.1±0.86^{BCa}	15.48±1.32^{ABa}	15.94±1.78^{Aab}	12.9±2.56^{ab}
	LE 2669	10.9±2.02^{Aab}	10.75±2.02^{Aab}	11.69±2.6^{Aa}	11.52±1.39^{Aa}	14.11±1.59^{Aa}	14.45±1.66^{Aab}	12.24±2.28^{b}
	LE 2511	10.2±1.64^{Cabc}	11.25±2.02^{BCab}	10.87±1.46^{Ca}	13.13±1.57^{ABCa}	15.78±0.02^{Aa}	14.25±0.79^{ABa}	12.44±2.34^{ab}
	Average	10.03±2.26^{C}	11.13±1.81^{BC}	11.60±2.12^{BC}	12.22±1.97^{B}	14.02±2.47^{A}	14.84±2.19^{A}	12.29±2.64
Red clover	LE 1731	14.36±2.08^{Aa}	14.07±2.28^{Aa}	14.36±0.44^{Aa}	17.01±0.43^{Aab}	18.85±1^{Aa}	15.89±2.51^{Aa}	15.61±2.28^{a}
	LE 1423	10.82±1.28^{Bb}	13.92±1.55^{ABa}	13.01±1.43^{ABab}	13.43±1.55^{ABbc}	14.88±1.41^{Aab}	14.67±0.43^{Aa}	13.39±1.8^{cd}
	LE 1391	9.61±2.01^{Cb}	13.92±1.91^{ABCa}	11.96±0.78^{BCabc}	17.88±2.61^{Aa}	16.24±2.92^{ABab}	16.77±3.52^{ABa}	14.4±3.67^{b}
	LE 2750	10.74±2.98^{Bb}	12.07±1^{ABab}	11.59±0.42^{ABbc}	13.58±2.08^{ABbc}	15.77±0.91^{Aab}	15.27±1.66^{Aa}	13.06±2.43^{de}
	LE 1599	10.25±1.32^{Bb}	11.47±1.73^{ABab}	11±2.15^{ABbc}	13.86±1.67^{Abc}	13.85±1.88^{ABb}	14.26±1.17^{Aa}	12.39±2.2^{e}
	LE 1775	12.22±2.31^{Bab}	12.33±1.55^{Bab}	13.22±1.18^{ABab}	14.34±0.99^{ABabc}	17.18±2.33^{Aab}	15.6±2.17^{ABa}	14.15±2.45^{bc}
	LE 1804	9.46±1.47^{Bb}	13.06±2.39^{ABab}	11.85±0.93^{ABabc}	12.81±2.99^{ABc}	16.31±0.98^{Aab}	15.91±2.02^{Aa}	13.24±2.95^{de}
	LE 1937	9.21±2.4^{Db}	11.18±0.94^{BCDb}	10.27±0.53^{CDc}	14.67±1.74^{ABabc}	13.48±1.44^{ABCb}	16.53±3.57^{Aa}	12.33±3.08^{e}
	Average	10.65±2.35^{C}	12.75±1.88^{BC}	12.16±1.59^{C}	14.45±2.56^{AB}	16.14±2.65^{A}	15.38±2.41^{A}	13.56±2.81
Average		10.34±2.31^{D}	11.95±2.01^{C}	11.88±1.88^{C}	13.33±2.53^{B}	15.08±2.76^{A}	15.11±2.30^{A}	12.93±2.89

TSP = triple superphosphate; Ash = biomass ash; Manure = cattle manure; Compost = biowaste compost; Average = average plant P uptake for each accession. Letters in capital case indicate significant difference between treatments (Tukey's test with $P < 0.05$). Letters in lower case indicate significant difference between accessions within same treatment (Tukey's test with $P < 0.05$).

The P concentration of the ground plant samples was measured after dry-ashing using the vanadate-molybdate method [2]. Plant P uptake was calculated as the product of plant P concentration and dry mass. The results were given as the sum in July and September.

3 Results and Discussion

Generally, a higher average P uptake was found in red clover than alfalfa ($P < 0.05$) (Table 2). On average of the crop species, the treatments with P amendment had significantly higher average P uptake than without and followed the order compost+TSP ≥ compost > manure > TSP ≥ ash > no P (Table 2), where compost and manure had higher average P uptake than TSP and ash ($P < 0.05$). Significant difference of plant P uptake between accessions was found in both alfalfa and red clover, but no significant interactions between treatments and accessions was found in either species. Cultivars of alfalfa had higher average P uptake than landraces, which might indicate a higher ability of alfalfa cultivars to utilise P from the soil. The higher P uptake of red clover landrace LE1731 in no P treatment implies a stronger P mobilisation than other accessions. To further evaluate the P efficiency, soil P tests will be conducted for both alfalfa and red clover. To verify the results, both species will be harvested and measured again in 2021.

References

[1] Homepage of National Inventory on Plant Genetic Resources in Germany, https://pgrdeu.genres.de/index.php/exsitu/suche, last accessed 2021/05/31.

[2] Page, A.L., Miller, R.H., Keeney, D.R. (1982): Methods of Soil Analysis. Chemical and Microbial Properties, 2nd edn. Soil Science Society of America, Madison, Wisconsin.

DOI: 10.5507/vup.21.24459677.10

A single genotype of the triploid hybrid *Festuca apennina* × *Festuca pratensis* expands over two hectares

Beat Boller[1] and David Kopecky[2]

[1] Langwiesstrasse 14, CH-8050 Zürich, Switzerland;
[2] Institute of Experimental Botany, Centre of Plant Structural and Functional Genomics Šlechtitelů 31,783 71 Olomouc – Holice, Czech Republic

beat.c.boller@bluewin.ch

Abstract. Plants of the sterile, rhizomatous, triploid (3x) *Festuca apennina × F. pratensis* hybrid were sampled on a predefined grid in two fields in the Swiss Alps. Cluster analysis of DArT markers revealed several distinct clusters of clonally replicated genotypes in one of the fields (Stoos), but no clustering in the other field (Gotthard). The largest clone at Stoos comprised 24 plants covering an area of 2.3 hectares with a maximum distance of 304 m between two plants. It is concluded that vegetatively propagated, sterile species hybrids can conserve heterosis and have the potential to produce a high yielding sward in mountain pastures.

Keywords: *Festuca,* interspecific hybrid, triploid

1 Introduction

The tetraploid (4x) grass species *Festuca apennina* De Not. frequently hybridizes with the diploid (2x) forage grass *F. pratensis* Huds. in nature to form sterile, rhizomatous, triploid (3x) hybrids [1, 2]. Such hybrids can also be obtained by simple bag crossings [3] and 3x progeny has been shown to exhibit extraordinary heterosis over their parental species [4]. Clonal replicates expanding for up to 14 m were identified among plants which were sampled in selected zones of a pasture heavily invaded by 3x hybrids, using DArT markers [1]. However, no attempt has been made so far to investigate the potential of a single genotype to dominate the sward of a pasture at a larger scale. Here, we compare two contrasting fields in the Swiss Alps with an important occurrence of 3x *F. apennina* × *F. pratensis* hybrids with respect to the spreading range of clonal replicates based on an analysis of DArT markers. The results show a great potential of single 3x genotypes to colonize large parts of a cattle grazed pasture.

2 Materials and Methods

Twelve pastures of approximately 2 ha were chosen at altitudes between 1350 and 1550 m in the Swiss Alps. About 50 plants of broad-leaved *Festuca* were collected on a pre-defined grid of 15 x 15 m as in [2]. Sampling points were georeferenced by GPS. Tillers were placed in boxes and allowed to grow in a greenhouse. Ploidy of each plant was determined by flow cytometry as in [2]. We chose two pastures with a dominant occurrence of 3x hybrids, Stoos (83 % hybrids) and Gotthard (67 % hybrids) for the detection of clonal replicates. DNA was extracted as in [1] and subjected to DArT marker analysis by the Diversity Array Technology Lab., Canberra (AUS). R software was used to run a cluster analysis on 4280 informative DArT markers according to Ward's method. Then, a distance matrix was constructed representing the percentage of markers differing between any pair of plants investigated. For some selected plants, maternity was determined by analysis of chloroplast DNA as in [1].

3 Results

The two pastures showed a completely different pattern of clustering among the 3x hybrids investigated. Plants from Stoos were grouped in highly distinct clusters comprising 2 to 24 individuals, while no clear grouping was evident for the Gotthard pasture (Fig. 1). For Stoos, analysis of pairwise distances between individuals showed an average 1.3 % (0.12 to 3.67 %) marker difference for plants within each cluster, considered to represent one genotype (clone), and a 17-fold higher (average 21.9 %; 12.0 to 34.2 %) marker difference for plants from different clusters (clones). For Gotthard, pairwise distances averaged 19.2 % (11.58 to 24.19 %) for all pairs of plants and thus, each plant represented an individual genotype.

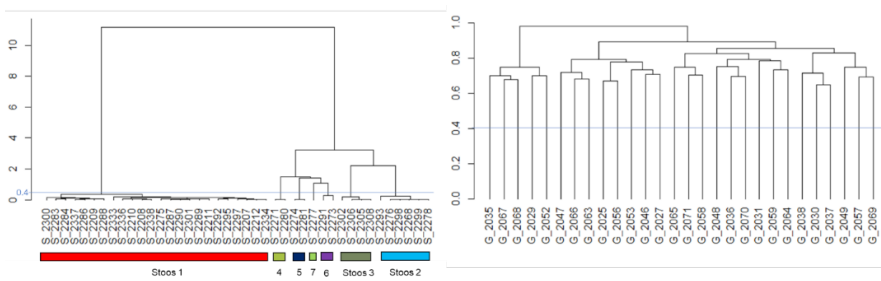


Fig. 1. Cluster analysis based on 4280 DArT markers determined on 41 (Stoos, left) and 28 (Gotthard, right) plants of triploid *F. apennina* x *F. pratensis*

The 24 plants belonging to the largest clone (Stoos 1) were spread over the whole zone investigated at Stoos (Fig. 2). The longest distance between two plants of this clone was 304 m and the surface of the polygon formed by the extreme positions of this clone was 2.3 hectares. The second largest clone (Stoos 2) with 6 plants expanded for a maximum distance of 132 m and covered 0.36 hectares. These two clones were identified as deriving from a cross *F. pratensis* (mother) × *F. apennina* (father). Two clones with 2 and 4 plants derived from a cross *F. apennina* (mother) × *F. pratensis* (father).

4 Discussion

Our results reveal a high potential of the sterile triploid hybrid *F. apennina* × *F. pratensis* to outcompete companion species in a natural pasture and to expand over larger areas than thought previously. The maximum distance between two clonal replicates of 304 m is more than 20 times longer than the maximum distance of 14.4 m reported so far [1]. One can just speculate why such a huge clone could develop in the field at Stoos, along with a number of smaller clones, but none developed in the field at Gotthard. Grazing by cattle only at Stoos, as opposed to yearly alternating cycles of cutting for hay and grazing at Gotthard may contribute. Also, hybrids may exist for a longer time at Stoos, allowing for wider expansion.

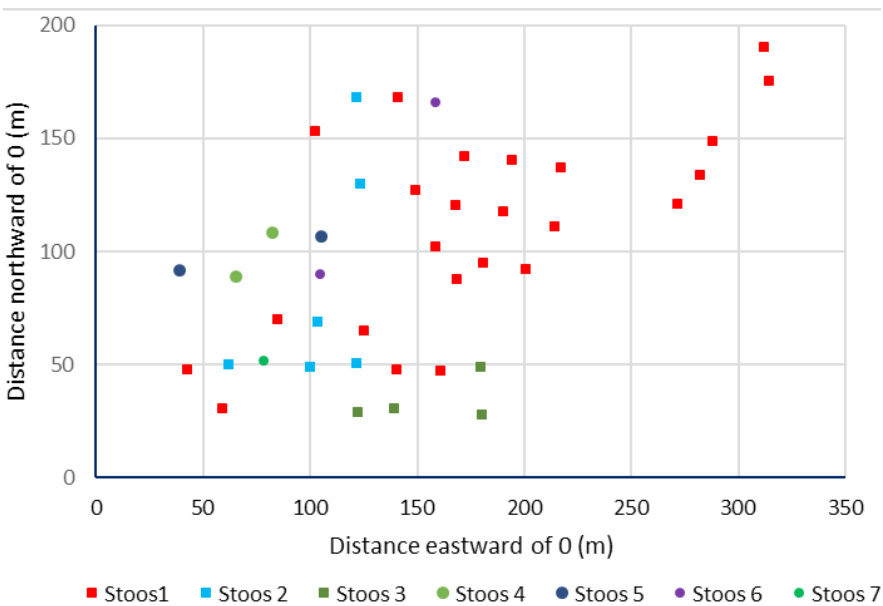


Fig. 2. Position of sampled plant of triploid hybrids *F. apennina* x *F. pratensis* at Stoos. Groups of clonal replicates are indicated with a common color and shape. WGS of 0: 46 °58’ 24” N / 8 ° 41’ 11” E.

A previous study has shown very high levels of heterosis when comparing 3x hybrids to their parents, *F. apennina* and *F. pratensis* [4]. Natural selection among 3x hybrids, over the years, may result in domination success of the best hybrid combinations, conserving heterosis by vegetative propagation.

The two largest hybrid clones identified at Stoos both derive from a *F. pratensis* mother. Overall, 3x hybrids from a *F. pratensis* mother occur less frequently in nature [1] and are obtained at an even lower rate when performing bag crosses [3] than 3x hybrids from a *F. apennina* mother. It thus appears that 3x hybrids from a *F. pratensis* mother are more competitive.

Highly competitive and productive, sterile 3x grass hybrids are candidates to exploit heterosis in an extraordinary way. Their potential as forage grasses is exemplified here by the successful colonization of a large pasture by a single hybrid genotype. It seems promising to seek for ways to propagate such particular genotypes vegetatively for use in mountain agriculture.

References

[1] Kopecký, D., Felder, T., Schubiger, F.X., Mahelka, V., Bartos, J., Dolezel, J., Boller, B. (2018): Frequent occurrence of triploid hybrids Festuca pratensis x F. apennina in the Swiss Alps. Alpine Bot. 128, 121–132.

[2] Boller, B., Harper, J., Willner, E., Fuchs, J. Glombik, M. et al. (2020): Spontaneous natural formation of interspecific hybrids within the Festuca-Lolium complex. Biol. Plant. 64, 679–691.

[3] Boller, B., Felder, T., Kopecky, D. (2018): Tetraploid Festuca apennina is prone to produce triploid hybrid progeny when crossed with diploid Festuca pratensis. In: Brazauskas, G., Statkevičiute, G., Jonavičiene, K. (eds.): Breeding Grasses and Protein Crops in the Era of Genomics. Pp. 33–38. Springer International Publishing Switzerland, Cham.

[4] Boller, B., Kopecky, D. (2020): Triploid forage grass hybrids Festuca apennina x pratensisdisplay extraordinary heterosis for yield characteristics. Euphytica 216, 143.

Session 2: Characterizing genetic diversity – the basis for selection

 DOI: 10.5507/vup.21.24459677.11

Understanding forage grass genomes beyond single nucleotide variation – an example on self-incompatibility

Bruno Studer[1][0000-0001-8795-0719], Marius Rohner[1], Chloé Manzanares[1] and the International *Lolium-Festuca* Pangenome Consortium[2]

[1] Molecular Plant Breeding, Institute of Agricultural Sciences, ETH Zurich, Universitaetstrasse 2, 8092 Zurich, Switzerland
[2] The International Lolium-Festuca Pangenome Consortium (ILFPC), including the following authors and affiliations:
Teagasc, Ireland, represented by Stephen Byrne
LAMMC, Lithuania, represented by Gintaras Brazauskas
DLF Seeds A/S, Denmark, represented by Christian Sig Jensen
LfL, Germany, represented by Stephan Hartmann
AgResearch, New Zealand, represented by Jeanne Jacobs
Norwegian University of Life Sciences, Norway, represented by Odd Arne Rognli
Agroscope, Switzerland, represented by Christoph Grieder
Aarhus University, Denmark, represented by Torben Asp
ETH Zurich, Switzerland, represented by Bruno Studer

Abstract. The genera *Lolium* and *Festuca* comprise the most important forage and turf grass species of temperate regions worldwide. Despite tremendous advancements in DNA sequencing technologies and genome assembly methods, genome sequences of highest quality in terms of completeness, correctness and contiguity are yet to be established for the *Lolium-Festuca* species complex. The International *Lolium-Festuca* Pangenome Consortium (ILFPC) has set out to fill this gap and aims at establishing multiple high-quality genome sequences to advance forage and turf grass research and breeding.

Here we report about first results of the joint efforts within ILFPC and, more importantly, how the established genomic resources can help answering biological questions. Using self-incompatibility (SI) as an example, we present how whole genome assemblies can be used to resolve the gene content and order at one SI locus, previously identified by fine-mapping. The multiple genomes obtained through ILFPC also enabled us to compare the gene constitution of different genotypes within and across species and link this information to SI functionality.

The availability of multiple high-quality genome assemblies constitutes a milestone for genetic studies, functional biology and genomics-assisted breeding. Moreover, comparing genomes and moving towards pangenomics opens new opportunities to describe structural genome variation and unlock genetic diversity in the *Lolium-Festuca* species complex.

Keywords: *Lolium-Festuca* species complex, International *Lolium-Festuca* Pangenome Consortium (ILFPC), Self-incompatibility.

1 Introduction

More than a century ago, Charles Darwin recognised the inability of plants to reproduce by self-pollination, a phenomenon known as self-incompatibility (SI). Since then, we have learned much about the genetic control of different SI systems that have evolved independently in different plant families. In the grass family (Poaceae), which is one of the largest plant families containing representatives of major forage and turf grass species, the SI mechanism remains elusive. Poaceae SI, unlike other systems, is governed by at least two multi-allelic, independent loci, *S* and *Z*. Map-based cloning of the *S*-locus in perennial ryegrass (*Lolium perenne* L.) identified the pollen component involved in the initial recognition during the SI reaction, a gene belonging to the DUF247 protein domain-containing gene family (hereinafter referred to as *LpSDUF247*) [1]. Similar to *S*, two independent mapping populations with more than 10^4 individuals segregating for *Z* were used to pinpoint the *Z* locus to a single BAC clone, containing five positional candidate genes (manuscript in preparation). However, none of the genes identified at *Z* have been unequivocally assigned to SI. The aim of this study was to further validate the *Z* locus region, by comparing the haplotypes of several genotypes of the *Lolium-Festuca* species complex.

2 Material and Methods

The perennial ryegrass haplotype containing the *Z* locus (BAC clone P205) was compared with the orthologous genome region from Byrne et al [2] (P226) and the doubled haploid perennial ryegrass genotype Kyuss [3]. Whole genome assemblies of a *L. multiflorum* Lam., Festulolium and *Festuca pratensis* Huds. genotype were established within ILFPC following the method described by Copetti et al. [4]. Genome sequences of *Dactylis glomerata* L. were taken from Huang et al. [5]. For synteny map construction, the gene models from Kyuss and *L. multiflorum* Lam. were used to identify the orthologous gene sequences. The presence and position of a specific gene in each contig, scaffold, or pseudomolecule were stored as coordinates in a CMAP format. The SimpleSynteny platform [6] was used to process each CMAP file individually and to illustrate a synteny map between the different genotypes and species.

3 Results and Discussion

The genomic constitution of the *Z* locus region previously established for perennial ryegrass (BAC clone P205) was confirmed in other genotypes of the *Lolium-Festuca* species complex (Figure 1). Gene content and order were conserved within genotypes exhibiting a functional SI system. A gene encoding for a protein containing a "domain of unknown function 247" is present in duplicate within the *Z* locus (*LpZDUF247A* and *LpZDUF247B*). Like for *LpSDUF247* at the *S* locus, the *LpZDUF247A* and *LpZDUF247B* genes were highly diverse between perennial ryegrass genotypes but also across the different Poeae species. In combination with flanking markers from fine-mapping, they constitute prime candidates for involvement in initial pollen-stigma recognition of a functional SI response. A continuous and complete haplotype reconstruction as well as haplotype-based diversity measurements were only possible with the assembled high-quality genome sequences obtained through ILFPC. In Manzanares et al. [1], despite the same initial fine-mapping and BAC clone sequencing approach, the *S* locus region was not complete, hindering the discovery of the female S component.

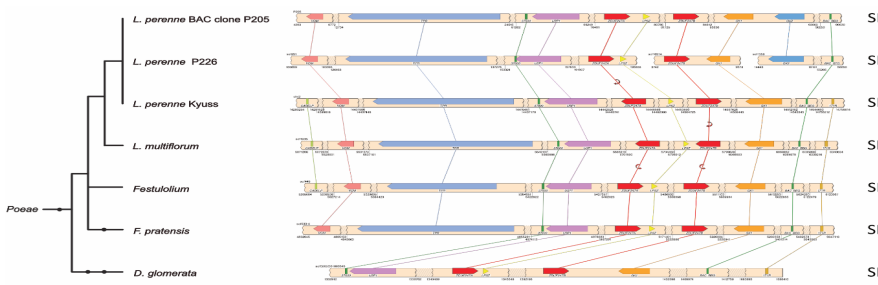


Fig. 1. Synteny map of the *Z* locus between multiple self-incompatible perennial ryegrass genotypes and species of the Poeae tribe. Each gene is represented by a uniquely colored arrow, indicating the direction of the coding sequence. Markers (37600, BAC_BEG, dark green) selected from the fine-mapping are displayed as uniquely colored rectangles and represent the flanking markers of the *Z* locus region.

References

[1] Manzanares, C., Barth, S., Thorogood, D., Byrne, S. L., Yates, S, Czaban, A., Asp, T., Yang, B., Studer, B. (2016): A gene encoding a DUF247 domain protein co-segregates with the *S* self-incompatibility locus in perennial ryegrass. Mol Biol Evol 33(4), 870–884 (2016)

[2] Byrne, S. L., Nagy, I., Pfeifer, M., Armstead, I., Swain, S., Studer, B., Mayer K., Campbell J. D., Czaban A., Hentrup S., Panitz F., Bendixen C., Hedegaard J., Caccamo M., Asp, T. (2015): A synteny-based draft genome sequence of the forage grass *Lolium perenne.* Plant J. 84(4), 816–826

[3] Frei, D., Veekman, E., Grogg, D., Stoffel-Studer, I., Morishima, A., Shimizu-Inatsugi, R., Yates, S., Shimizu, K. K., Frey, J. E., Studer, B., Copetti, D.: Ultralong Oxford Nanopore reads enable the development of a reference-grade perennial ryegrass genome assembly. Genome Biol Evol, *in press*

[4] Copetti, D., Yates, S., Vogt, M. M., Russo, G., Grieder, C., Kölliker, R., Studer, B. (2021): Evidence for high intergenic sequence variation in heterozygous Italian ryegrass (*Lolium multiflorum* Lam.) genome revealed by a high-quality draft diploid genome assembly. bioRxiv

[5] Huang, L., Feng, G., Yan, H., Zhang, Z., Bushman, B. S., Wang, J., Bombarely A., Wang, J., Bombarely A., Li M., Yang Z., Nie G., Xie W., Xu L., Chen P., Zhao X., Jiang W., Zhang X. (2020): Genome assembly provides insights into the genome evolution and flowering regulation of orchardgrass. Plant Biotechnol J 18(2), 373–388

[6] Veltri, D., Wight, M. M., Crouch, J. A.: SimpleSynteny (2016): a web-based tool for visualization of microsynteny across multiple species. Nucleic Acids Res 44(W1), W41–W45

 DOI: 10.5507/vup.21.24459677.12

Climate change adaptation in forage grasses: from phenotype to genotype

Kristina Jaškūnė[1] and Gražina Statkevičiūtė[1]

[1] Lithuanian Research Centre for Agriculture and Forestry, Instituto av. 1, 58344 Akademija, Lithuania

kristina.jaskune@lammc.lt

Abstract. Precise, non-invasive and high-throughput phenotyping methods are a crucial part for the identification of genes underlying plant adaptivity to abiotic stresses and for transferring this knowledge to practical breeding. Baltic and Nordic regions of Europe are facing an increased frequency of mild summer droughts. Therefore, securing stable forage production by improving grass tolerance to water deprivation is a pressing issue. Dissecting biomass accumulation by precise phenotyping of leaf growth under optimal and drought stress conditions, associating adaptive traits with candidate genes and subsequently validating their functions by employing CRISPR-Cas based genome editing, can be an efficient strategy to increase sustainability in agriculture through improvement of perennial ryegrass adaptation to current and future climates.

Keywords: perennial ryegrass, growth, drought

1 Introduction

Permanent grassland and meadows make up one-third of the agricultural land in the EU [1], forming an instrumental input for livestock farming. The growing number of beef cattle farms demands increasing amounts of high-quality pastures, but at the same time need to adapt to new environmental and economical requirements and restrictions. These require maximizing production per unit area with less input of water, fertilizers and pesticides leading to lower environmental footprints including GHG emissions and resulting in a fair, healthy and environmentally friendly food system. Global warming offers a possibility to increase productivity in the Baltic region as the higher average temperatures, extended growing seasons and longer frost-free periods may result in improved yield and reduced carbon footprint due to higher yields and better feed efficiency [2]. On the other hand, the predicted climate shifts increase frequency of dry periods which may impose on grassland productivity leading to huge yield losses and thus escalated fodder demand [3,4]. Mild drought, which is typical

for temperate environments, does not threaten survival of the crops, however, it significantly reduces yield. Therefore, applied research that addresses future demands of forages in response to climate change and supports sustainable agriculture is receiving much attention. In this paper we summarized our recent year achievements in perennial ryegrass breeding for biomass formation under drought using novel phenotyping and genotyping methods.

2 Phenotyping a Dynamic Trait

Superior feed quality and productivity makes perennial ryegrass (*Lolium perenne* L.) the predominant forage grass species in Western Europe. However, the main limitation of perennial ryegrass wider distribution further north and east is its poor performance under unfavourable conditions. Water limitation is one of the major factors reducing the yield of crops in temperate regions and worldwide [5]. Despite increasing interest in research on mechanisms causing yield losses under water deficit, such studies proved to be difficult and not very successful [6]. A limitation is that yield often is measured destructively at the end of the experiment and in this way the dynamic process of growth is overlooked. As the biomass accumulation is largely determined by leaf growth, phenotyping leaf elongation in response to water deficit might give a hint in what directions we should breed. Using a non-destructive, high-throughput and non-labor intensive method to monitor real time leaf growth under adverse environmental conditions [7] we have revealed that leaf elongation under stress conditions is not linear but can be described by three phases: the first phase describes growth in response to temperature, in the second phase the plant decelerates leaf elongation proportionally to soil water potential, and finally, leaf growth terminates at the third phase [8]] Additionally, the Tri-Phase model enabled us to determine points which demarcate the phases thus they can be used to describe the response of plants to water deficit. For accurate quantification of plant tolerance to water deficit, we have used this dynamic phenotyping method in a perennial ryegrass association panel and revealed high variation of leaf elongation traits with no apparent clustering of genotypes representing turf or forage type cultivars and natural ecotypes ([9] (Fig. 1). The most desirable trait combination for a forage cultivar is fast growth under optimal conditions, paired with moderately early slow down under drought stress, but late growth arrest, i.e., high tolerance, allowing the crop to produce biomass regardless of unfavourable weather conditions. Forage-type genotypes tended to grow faster under optimal conditions, especially compared to turf-type genotypes [9]. However, some of the forage-type genotypes were located at the negative coordinates of PC1 and PC2 and were thus associated with low values of leaf elongation under optimal and stressed conditions (Fig. 1). This indicates, that there is still plenty of room for improvement in forage breeding.

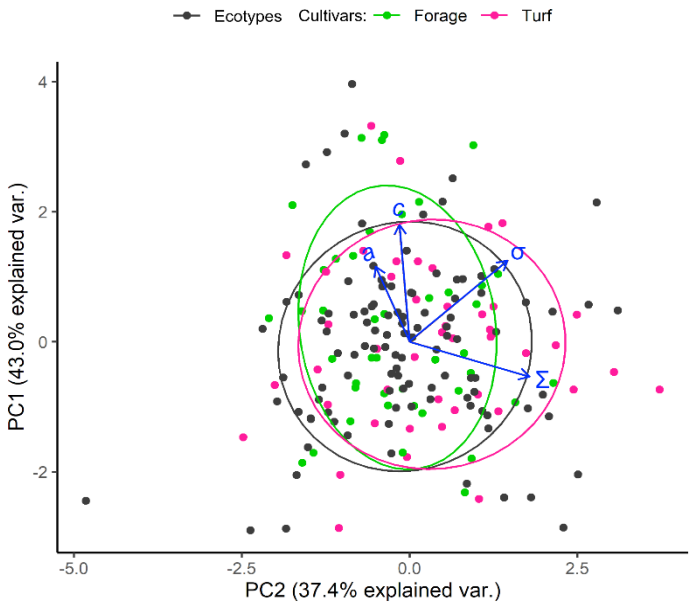


Fig. 1. Principal component analysis (PCA) biplot of 197 perennial ryegrass cultivars and ecotypes based on leaf growth traits: thermal leaf growth (*a*), leaf growth reduction point (Σ), leaf growth arrest point (σ), and water deprivation tolerance (*c*).

3 Phenotype to Genotype

The breathtaking progress in genomic technologies allowed fast and relatively cheap accumulation of vast amounts of genotypic data, while plant phenomics lagged behind. Recent development of imaging, sensor technologies and data analysis provide endless opportunities for in-depth studies of molecular mechanisms behind various plant physiological processes and to accelerate breeding for stress-tolerant crops [10]. Agriculture is traditionally considered an important part of economy in Baltic countries, however, the breeders rely mainly on the classical approach which is a time-consuming process. Lacking the implementation of state-of-art phenotyping and genotyping methods and strategies will result in lagging behind the economies/companies which apply modern breeding methods. In addition, the scarcity of functional genomics studies in perennial ryegrass hampers the understanding of the role of specific genes which can be utilized to develop cultivars with improved adaptation. Applications in breeding require linking genes to specific traits and validating their role and recently emerged genome editing technology offers the approach to achieve this. Drought, as well as other abiotic stress resistance is governed by a complex network of genes, however, the major players are relatively well established and proposed as possible targets for CRISPR/Cas9 editing [12]. The two candidate genes with

well-known roles in stress resistance, *phytochrome B* and *MYB* transcription factor, were associated with leaf growth under water deficit in perennial ryegrass, making them primary candidates as well [9]. A first report on genome editing in perennial ryegrass was published recently [13]; however, transformation efficiency was relatively low and still far from routine. The newly launched project EditGrass4Food aims at performing functional studies of gene variants using gene editing (CRISPR) technologies. We intend to utilize unique pre-breeding material and CRISPR-based editing to validate candidate genes involved in northern adaptation of perennial ryegrass. We will focus on genes involved in the mechanisms of freezing tolerance and biomass growth under water deficit. Moreover, we will investigate changes during abiotic stress periods at the transcriptome level to reveal gene regulatory pathways and networks. This will enable us to utilize the gained information in future genomic selection programs to develop ryegrass cultivars with improved freezing and drought tolerance and persistence. It will also help breeders and agriculture in general in the Nordic/Baltic region to prepare for meeting new demands due to climate change and changing societal demands.

Acknowledgements

We sincerely thank Prof. Bruno Studer, dr. Steven Yates for the remarkable work in developing phenotyping system which the paved the way for further research. We further thank Prof. Odd Arne Rognli, dr. Nils Rostoks and dr. Cecilia Sarmiento for collaboration in "Improving adaptability and resilience of perennial ryegrass for safe and sustainable food systems through CRISPR-Cas9 technology - EditGrass4Food", project no. EEA-RESEARCH-64.

References

[1] Eurostat, No Title, (2014): http://ec.europa.eu/eurostat.

[2] J.E. Olesen, M. Trnka, K.C. Kersebaum, A.O. Skjelvåg, B. Seguin, P. Peltonen-Sainio, F. Rossi, J. Kozyra, F. Micale (2011) Impacts and adaptation of European crop production systems to climate change, Eur. J. Agron. 34 96–112. https://doi.org/10.1016/j.eja.2010.11.003.

[3] S. Daryanto, L. Wang, P.-A. Jacinthe (2016): Global Synthesis of Drought Effects on Maize and Wheat Production., PLoS One. 11 e0156362. https://doi.org/10.1371/journal.pone.0156362.

[4] S. Fahad, A.A. Bajwa, U. Nazir, S.A. Anjum, A. Farooq, A. Zohaib, S. Sadia, W. Nasim, S. Adkins, S. Saud, M.Z. Ihsan, H. Alharby, C. Wu, D. Wang, J. Huang (2017): Crop Production under Drought and Heat Stress: Plant Responses and Management Options, Front. Plant Sci. 8 1–16. https://doi.org/10.3389/fpls.2017.01147.

[5] F.C. Moore, D.B. Lobell (2015):The fingerprint of climate trends on European crop yields, Proc. Natl. Acad. Sci. 112 2670–2675. https://doi.org/10.1073/pnas.1409606112.

[6] H. Nelissen, M. Moloney, D. Inzé (2014): Translational research: from pot to plot., Plant Biotechnol. J. 12 277–85. https://doi.org/10.1111/pbi.12176.

[7] K. Jonavičienė, S. Yates, S. Nagelmüller, F. Liebisch, N. Kirchgessner, A. Walter, G. Brazauskas, B. Studer (2016): Real time growth analysis of perennial ryegrass, in: Breed. a World Scarcity, Springer pp. 105–110.

[8] S. Yates, K. Jaškūnė, F. Liebisch, S. Nagelmüller, N. Kirchgessner, R. Kölliker, A. Walter, G. Brazauskas, B. Studer (2019): Phenotyping a Dynamic Trait: Leaf Growth of Perennial Ryegrass Under Water Limiting Conditions, Front. Plant Sci. 10 1–9. https://doi.org/10.3389/fpls.2019.00344.

[9] K. Jaškūnė, A. Aleliūnas, G. Statkevičiūtė, V. Kemešytė, B. Studer, S. Yates (2020): Genome-Wide Association Study to Identify Candidate Loci for Biomass Formation Under Water Deficit in Perennial Ryegrass, Front. Plant Sci. 11 570204. https://doi.org/10.3389/fpls.2020.570204.

[10] S.L. Byrne, I. Nagy, M. Pfeifer, I. Armstead, S. Swain, B. Studer, K. Mayer, J.D. Campbell, A. Czaban, S. Hentrup, F. Panitz, C. Bendixen, J. Hedegaard, M. Caccamo, T. Asp (2015): A synteny-based draft genome sequence of the forage grass Lolium perenne, Plant J. 84 816–826. https://doi.org/10.1111/tpj.13037.

[11] Y. Zhang, Y. Ran, I. Nagy, I. Lenk, J.-L. Qiu, T. Asp, C.S. Jensen, C. Gao (2020): Targeted mutagenesis in ryegrass (Lolium spp.) using the CRISPR/Cas9 system, Plant Biotechnol. J. 18 1854–1856. https://doi.org/10.1111/pbi.13359.

DOI: 10.5507/vup.21.24459677.13

Development of a metabolic profiling method for ryegrass phenotyping and breeding on drought tolerance

Johannes Wittmann[1], Peter Westermeier[2], Evelin Willner[3], Stephan Hartmann[2] and Roland Geyer[1]

[1] lifespin GmbH, Am Biopark 13, D-93053 Regensburg
[2] Institute for Crop Science and Plant Breeding, Bavarian State Research Center for Agriculture, Am Gereuth 4, D-85354 Freising
[3] Satellite Collections North, Genebank Department, Leibniz Institute of Plant Genetics and Crop Plant Research, Inselstr. 9, D-23999 Malchow / Insel Poel

roland.geyer@lifespin.de

Abstract. The project DRYeGRASS focused on perennial ryegrass (*Lolium perenne* L.), as one of the most important forage grass species. One goal of the project was to efficiently improve tolerance to temporary drought using innovative selection methods. Therefore, a nuclear magnetic resonance (NMR)-based metabolic profiling method was developed. The resulting method provides quantitative metabolite data in sufficient quality and is still affordable for breeders. It combines a highly automated process from sampling to the final processed NMR spectrum and a profiling software that was developed in the project. This software produces a quantitative profile comprising 48 metabolites. Using this method >1400 individual plant samples from a two-year trial at two locations were analysed. Finally, the resulting metabolite profiles were used to train a prediction model using machine learning. This model enables the selection for tolerance to temporary drought at an early stage of a breeding process. In summary, a useful complementary method for phenotyping was developed and exemplarily applied for biomarker research in the breeding on drought tolerance.

Keywords: Metabolic profiling, metabolite biomarker, drought stress.

1 Introduction

Phenotypic information is an essential part of plant science and breeding. But, in many cases it is also the bottleneck, e.g., due to missing efficiency in the quantification of metabolite concentrations. Nuclear magnetic resonance (NMR)-based metabolic profiling, including software for automated metabolite identification and quantification, is a well-suited tool to solve this contradiction. Thus, in the project DRYeGRASS, such a system for multiparameter quantification was de-

veloped for perennial ryegrass (Lolium perenne L.). Furthermore, the project focused on tolerance of ryegrass to temporary drought, and the developed method was used as a basis to develop a prediction model that enables the selection for tolerance to temporary drought, without the necessity of performing drought stress experiments. To that end, the resulting metabolic profiles of >1400 single plants were correlated with phenotypic data obtained in a randomized two-location rain-out shelter experiment.

2 Experimental Design

Phenotypic data for drought tolerance were obtained for eight segregating, connected crossing populations in a randomized two-location rain-out shelter experiment (Malchow/Poel and Freising). During vegetation periods of 2017 and 2018, watering was withheld twice a year for up to six weeks to induce drought stress. After cutting of the plants, a well-watered period of 4–6 weeks followed until next cutting date. Comprehensive phenotypic data were collected at several time points over the two years and provided for correlation with the metabolic profiles.

For the metabolic profiling sampling of leaves of >1400 individual plants (including control genotypes and replicates) was performed 10 days after the first cut in both years, thus before the first stress was induced. It was performed in tight and comparable timelines (max. 2 h, before 12 am) and provided 200–300 mg fresh matter of at least three 1–2 cm leaf cuts per plant. The samples were immediately frozen on dry ice and lyophilized overnight. For method development all subsequent process steps were investigated and optimized. For the automated identification and quantification of metabolites from the resulting NMR spectra a software for signal deconvolution and curve fitting was developed. This software was combined with a reference spectra database comprising > 200 metabolites. The resulting method is summarized below.

Data analysis was done based on these leaf metabolite profiles and phenotypic data from the field experiments. For the development of the prediction model combinations of metabolite concentrations were correlated with membership function values of drought stress tolerance that combine adjusted means of several relevant single scores from the rain-out shelter experiments. Therefore, Random Forest models were calculated in a nested cross-validation with 10 folds and 5 repeats each. The outer loop was used for error-estimation. As a performance measure for the error, we used the area under the receiver operating characteristic curve (ROC-AUC). The inner loop was used to extract the 10 best features with recursive feature elimination.

3 Results and Discussion

The developed system for metabolic profiling combines optimized sample preparation processes, automated 1D 1H NMR-measurement and software-based metabolite quantification. Optimization of the sample preparation protocol resulted in homogenizing with a ball mill and subsequent extraction with an aqueous buffer solution for 20 min at 85°C and 40 min cool down time at room temperature. After centrifugation the supernatant is mixed (90:10 v/v) with additives solution containing D2O and internal standards. The NMR measurement is performed using a Bruker Avance HD III 600 MHz spectrometer with sample jet autosampler. 600 MHz spectrometer provide the ideal compromise between costs and information/resolution in the spectrum. Resulting NMR spectra are then automatically processed (referencing, phase and baseline correction) and verified by inter- and intra-serial quality control routines comparing internal standard values. The developed profiling software is able to deconvolute superimposed NMR signals and quantifies a panel of 48 metabolites (amino acids, sugars, alcohols, amines, aldehydes, acids, …) against internal standards. The method enables the analysis of 96 samples per day.

The descriptive analysis of the data set (i.e., the quantitative metabolite profiles) showed differences of the metabolic profiles depending on, e.g., genotypes, locations, years, populations and other factors of the experimental design. In this short paper we can only give an example of this comprehensive information content of metabolic profiles and the resulting opportunities for research and breeding. In **Fig. 1** the dependency of selected metabolite concentrations on year and location is shown. Some metabolite concentrations appear to be stable (e.g. succinate), while others vary significantly (e.g. choline, GABA, glutamine, glycerol, lactate and valine).

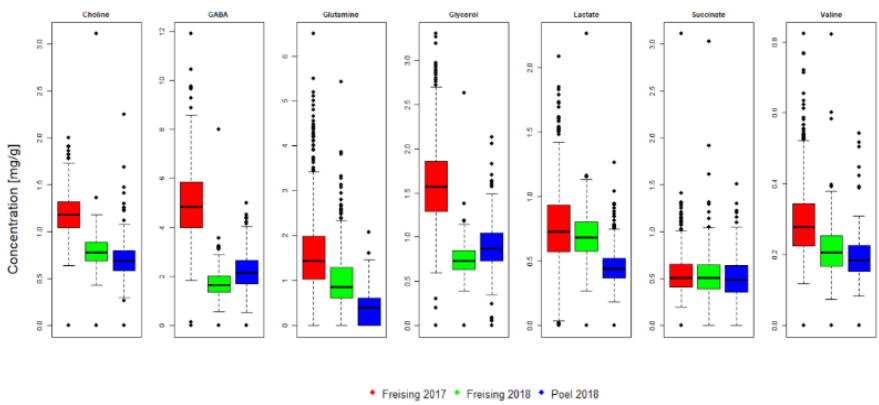


Fig. 1. Box plots of 7 selected metabolite concentrations showing dependency on year and location. The red boxes represent location 1 (Freising) in 2017 (n=678), green boxes represent location 1 in 2018 (n=304) and blue boxes represent location 2 (Poel) in 2018 (n=300).

Finally, the 48 metabolite concentrations were investigated in the context of drought tolerance and a prediction model was developed that can be used as a selection tool. We obtained a ROC-AUC of 0.73 with standard deviation 0.1 using the 10 best features. Training on all features we obtained a ROC-AUC of 0.77 with standard deviation 0.09. It is possible with this model to test for the 10% of the samples in an experiment that have the highest overall performance in terms of drought stress tolerance. The prediction accuracy for both groups (10% best and 90% remainder) is approx. 70%. This means that it is possible to select for tolerance to drought stress, based on metabolic profiles and at an early stage of a breeding process, without a drought stress experiment.

Acknowledgement

The research leading to these results received funding from the German Federal Ministry of Food and Agriculture (BLE Innovationsförderung; FKZ: 2818209615, DRYeGRASS).

DOI: 10.5507/vup.21.24459677.14

Pooled sequencing reveals genome regions involved in resistance to bacterial wilt in Italian ryegrass

Florian Goettelmann[1], Dario Copetti[1], Steven Yates[1], Bruno Studer[1] and Roland Kölliker[1]

[1] Molecular Plant Breeding, Institute of Agricultural Sciences, ETH Zurich, Universitaetstrasse 2, 8092 Zurich, Switzerland

roland.koelliker@usys.ethz.ch

Abstract. *Xanthomonas translucens* pv. *graminis* (Xtg) is the causal agent of bacterial wilt, one of the main diseases of Italian ryegrass (*Lolium multiflorum* Lam.). One major QTL for resistance was previously discovered, but the underlying genes are yet to be determined. In order to fine-map this QTL, a mapping population consisting of 7,484 F_2 individuals segregating for resistance was established in the greenhouse and inoculated with a highly virulent Xtg strain. Two pools of the most resistant and the most susceptible individuals were sequenced and SNPs associated with resistance were identified. Most of the significant SNPs map to linkage group 4, where the QTL was previously identified. Genes containing these SNPs will be determined and will constitute candidate resistance genes to be investigated further.

Keywords: Bacterial wilt, disease resistance, Italian ryegrass, *Lolium multiflorum* Lam., pooled sequencing, *Xanthomonas translucens* pv. *graminis*,

1 Introduction

Xanthomonas translucens pv. *graminis* (Xtg), the causal agent of bacterial wilt of forage grasses, is one of the most important pathogens of Italian ryegrass (*Lolium multiflorum* Lam.), causing serious yield and quality losses [1]. Cultivars with partial resistance to Xtg are available, however, since *L. multiflorum* is an allogamous species, cultivars have a high level of heterozygosity, and susceptibility still occurs in these cultivars. Marker-assisted selection would be greatly beneficial for selection and fixation of resistance alleles in *L. multiflorum* to breed resistant cultivars more rapidly and more efficiently.

Using an F_1 mapping population derived from a cross between a resistant and a susceptible parent (hereafter referred to as the Xtg-ART population), previous work identified a QTL explaining 43 to 84 % of resistance to Xtg on linkage group (LG) 4 [5]. However, further characterization of the QTL and the development of markers was hindered by the lack of sequence information for *L. multiflorum*

at the time. More recently, a pooled sequencing approach using the Xtg-ART population allowed the identification of SNPs associated to this QTL [2]. This served as a proof-of-concept for the pooled sequencing approach, but was limited in its resolution due to the low sequencing coverage and the small number of individuals used in the analysis.

We aimed at fine-mapping this QTL at high resolution by using a pooled sequencing approach in a large F_2 population derived from Xtg-ART using next-generation DNA sequencing technology.

2 Materials and Methods

A total of 7,484 F_2 individuals derived from Xtg-ART were established in the greenhouse, and leaves from each plant were harvested individually. Plants were allowed to grow back and were then inoculated with the highly pathogenic strain Xtg29 [4].

Disease was monitored and scored on a scale from one to five at 14, 21, 28, and 49 days post inoculation, and the 750 most resistant individuals and the 761 most susceptible individuals were selected to form a resistant and susceptible pool, respectively. Both pools were randomly divided into three subpopulations of an equal number of samples. DNA was extracted from the previously harvested leaves and libraries for each subpopulation were prepared for whole genome sequencing using Illumina TruSeq Nano DNA Library Preparation, (Illumina, San Francisco, CA, USA). Libraries were then sequenced using 150 bp paired-end reads on a NovaSeq 6000 S4 flowcell.

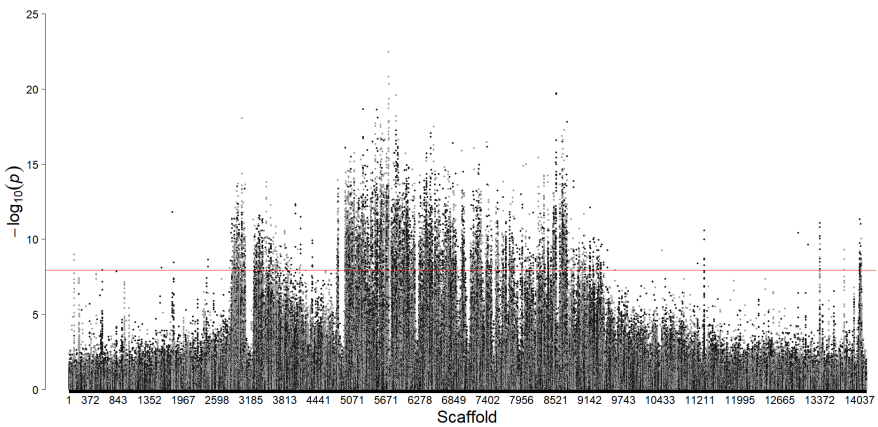


Fig. 1. Association between SNPs and resistance on linkage group 4. The X-axis shows each scaffold ordered based on its relative position on the genome sequence of barley (*Hordeum vulgare* L.). The Y-axis shows the -$\log_{10}$ of the p-value associated with each SNP. The red line indicates the significance threshold after Bonferroni correction.

Reads were mapped on the genome sequence of M2289, the resistant parent of the Xtg-ART population [3], and SNPs were determined using bcftools 'call' from a samtools 'mpileup'. After filtering for low (<50) and high (>400) read counts, and quality (>50 QUAL score), association between each SNP and resistance or susceptibility was tested by Cochran Mantel Haenszel test.

3 Results and Discussion

A total of 602,742 Gbp were obtained by sequencing (average = 100,457 Gbp per subpopulation), corresponding to an average of 44X coverage of the haploid genome per subpopulation. After mapping the reads to the M2289 genome sequence, 8,435,414 SNPs were identified, and 4,366,368 remained after filtering. The Cochran Mantel Haenszel test revealed 12,933 significant SNPs after Bonferroni correction. Most of these were located on LG 4, corresponding to the previously identified QTL (Fig. 1).

4 Conclusion

Using a pooled sequencing approach, we were able to identify SNPs associated with resistance to Xtg. Most of these SNPs are located on LG 4, where the QTL for resistance was previously identified, confirming the reliability of this approach. Moreover, the high number of progenies used, together with the sequencing coverage obtained, resulted in higher marker resolution compared to the previous QTL analyses, which were based on a small number of markers. Further analysis of the data will allow to identify candidate genes within this region, which will, after validation, allow to better understand the mechanisms of the interaction and to develop genomics-assisted breeding strategies to improve Xtg resistance in *L. multiflorum*.

Acknowledgements

This research was supported by the Swiss National Science Foundation (Grant No: IZCOZO_177062)

References

[1] Egli T, Goto M, Schmidt D (1975) Bacterial wilt, a new forage grass disease. Journal of Phytopathology 82:111–121.

[2] Knorst V, Byrne S, Yates S, Asp T, Widmer F, Studer B, Kölliker R (2018) Pooled DNA sequencing to identify SNPs associated with a major QTL for bacterial wilt resistance in Italian ryegrass (Lolium multiflorum Lam.). Theoretical and Applied Genetics 132:947–958.

[3] Knorst V, Yates S, Byrne S, Asp T, Widmer F, Studer B, Kölliker R (2018) First assembly of the gene-space of Lolium multiflorum and comparison to other Poaceae genomes. Grassland Science 65:125–134.
[4] Kölliker R, Kraehenbuehl R, Boller B, Widmer F (2006) Genetic diversity and pathogenicity of the grass pathogen Xanthomonas translucens pv. graminis. Systematic and Applied Microbiology 29:109–119.
[5] Studer B, Boller B, Herrmann D, Bauer E, Posselt UK, Widmer F, Kölliker R (2006) Genetic mapping reveals a single major QTL for bacterial wilt resistance in Italian ryegrass (Lolium multiflorum Lam.). Theoretical and Applied Genetics 113:661–671.

DOI: 10.5507/vup.21.24459677.15

Genetic analysis of drought stress tolerance in perennial ryegrass (*Lolium perenne* L.)

Johann Huber[1], Peter Westermeier[1], Volker Mohler[1], Evelin Willner[2] and Stephan Hartmann[1]

[1] Bavarian State Research Center for Agriculture (LfL); Institute for Crop Science and Plant Breeding, Freising, Germany
[2] Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Genebank, Satellite Collections North, Malchow/Gross Luesewitz, Germany

Johann.Huber@lfl.bayern.de

Abstract. The objective of this study was to determine quantitative trait loci (QTL) for drought stress tolerance based on yield in perennial ryegrass. Therefore, 2190 single nucleotide polymorphism (SNP) markers were examined to construct linkage maps for three F_1 populations with 140 individuals each. Three highly drought stress tolerant genotypes, two from two advanced cultivars and one from a genebank accession, were used as parents. The total length of genetic maps ranged from 480 to 590 centimorgan (cM). Phenotypic data were collected in a rain-out shelter and a companion control plot in two locations. Genome-wide association studies (GWAS) were performed to determine significant marker-trait associations (MTA). Both cultivar genotypes inherited a major QTL located on LG1. This QTL can further be used for specific selection on drought stress tolerance in perennial ryegrass breeding programs.

Keywords: Perennial ryegrass, *Lolium perenne*, drought tolerance, linkage mapping, GWAS

1 Introduction

Biomass production becomes more and more difficult due to climate change and extreme weather events. Breeding for drought stress tolerance has high potential for ensuring food and biomass production. Especially the adaptation of perennial forage grass species to abiotic stress factors improves biomass security [1]. As drought stress tolerance has a low heritability, selection gain via traditional breeding methods is quite small [1] and marker-assisted selection (MAS) will be needed to obtain satisfying breeding results. In this study, three diploid *L. perenne* populations (DryeGrass diallel) were characterized for their reaction to drought stress [2] and used for genome-wide association studies (GWAS)-based quantitative trait loci (QTL) analysis in the present study.

2 Material and Methods

Plant material and field trial design: Three diploid perennial ryegrass genotypes, two from two cultivars from Deutsche Saatveredelung and one from accession GR 5559 of the IPK genebank, previously characterized as drought tolerant [2], were crossed through free fading in pollination bags to produce three F_1 populations with 140 genotypes each. The populations were named K03 (cultivar 1 × cultivar 2), K11 (cultivar 1 × accession) and K14 (cultivar 2 × accession). In this study, drought tolerance was defined as the capacity for a quick regrowth after drought. The field trials were conducted in Pulling/Freising (Southern Germany) and in Malchow/Poel (Northern Germany) in 2017 and 2018. The populations were grown in a field plot with natural precipitation and in a rain-out shelter with two induced drought periods per year in mid spring (after cut 1) and early summer (after cut 3) for 4–6 weeks respectively to reduce the volumetric soil water content to less than 10%. The field trials were randomized as an augmented block design with 35 F1-individuals and 5 out of 10 genotypes (including population parents) as standards in each block and 4 blocks per population. Each plot consisted of cloned plants from one genotype.

Phenotypic data: For each of the five cuttings, plant recovery after cut was visually scored on a scale from 0 (no biomass) to 9 (very strong biomass growth) and dry matter (DM) yield was determined by drying and weighing the plot yield. Data for cuttings 1, 3 and 5 (vegetation start and recovery after drought) were used for further analysis. Block effects were estimated with a mixed model and adjusted means were calculated. For visual scoring and DM yield data stress tolerance indices (STI) were calculated [3]. STIs were combined to membership function values of drought stress tolerance (MFVD) [4] for biomass scoring and DM separately as well as for both traits combined.

Linkage mapping and GWAS: 2190 codominant SNP markers were selected from a SNP array comprising 3681 SNP-markers [5] to genotype the three populations. 1189, 1128, 1139 of the SNPs were polymorphic in K03, K11 and K14, respectively. Genotypic data were generated by LGC Genomics (Berlin) using targeted genotyping by sequencing technology. Linkage maps were calculated with JoinMap 5 (Kyazma ®) using the regression method and the Haldane mapping function. Based on genotypic and phenotypic data GWAS were performed in R [6] with GenABEL [7]. Significant MTAs along LGs were merged to QTL regions. Estimates of r^2 between two trait-associated markers higher than the 95th percentile of the distribution of r^2 between a subset of unlinked markers in each population were considered to indicate genetic linkage [8].

3 Results and Discussion

In each population, SNP markers were distributed across 7 LGs corresponding to the basic chromosome number of diploid perennial ryegrass (Table 1). The total number of mapped markers in the populations ranged from 951 to 973. The highest number of markers across populations was assigned to LG4, whereas the lowest number of markers was found for LG5. In GWAS, significant MTAs for MFVD-indices were found in populations K11 and K14 (Table 2). After determining the number and genetic position of QTL, K11 and K14 were found to share a QTL located on LG1 for all traits (DM 2017 and 2018, scoring, DM and combined index; adj. means of 2 locations;). As this multi-trait QTL was inherited from both cultivar genotypes, it was not found in population K03. In population K11, additional QTL for DM yield in 2017 and 2018 were found on LGs 2, 3, 4 and 6 (data not shown).

Table 1: Summary statistics of the linkage maps divided by populations (horizontal) and linkage groups (vertical) for map length in centiMorgan (cM) and number of markers

	K03		K11		K14		mean	
LG	SNP markers	length [cM]	SNP markers	length [cM]	SNP markers	length [cM]	SNP markers	length [cM]
1	171	89.9	106	56.2	149	80.7	142	75.6
2	128	81.6	146	83.0	143	107.6	139	90.7
3	162	67.4	155	50.3	129	70.3	149	62.7
4	192	86.5	175	70.1	166	110.0	178	88.9
5	93	41.4	105	85.5	62	48.1	87	58.4
6	48	79.9	133	68.7	127	71.5	103	73.4
7	179	73.4	137	68.6	175	103.3	164	81.8
Total	973	520.2	957	482.4	951	591.5	960	531.4

Table 2: p-values of QTL peak markers for traits across locations in populations K11 and K14; one QTL was detected per LG; n.s. not significant

Population	Trait	LG1	LG3
K11	visual-scoring index (MFVD)	1.11E-10	0.0010
	DM index (MFVD)	5.08E-08	n.s.
	combined index (MFVD)	1.52E-10	n.s.
K14	visual-scoring index (MFVD)	6.16E-13	n.s.
	DM index (MFVD)	7.18E-10	n.s.
	combined index (MFVD)	7.73E-13	n.s.

4 Conclusion

The consistent multi-trait QTL on LG1 may provide a helpful tool to improve drought tolerance in perennial ryegrass by MAS in modern breeding programs.

References

[1] Wilkins P. W. and Humphreys M. (2003) Progress in breeding perennial forage grasses for temperate agriculture. *The Journal of Agricultural Science* 140, 129–150.
[2] Westermeier P., Willner E., Wosnitza A., Feuerstein U., Luesink W., Schulze S., Schum A. and Hartmann S. (2014) Genetische Variation des Merkmals Trockentoleranz in DeutschemWeidelgras (*Lolium perenne L.*) - Evaluierung phänotypischer Selektionsmerkmale. In: Brandstetter A., Geppner M., Grausgruber H. and Buchgraber K. (eds) 65. Tagung der Vereinigung der Pflanzenzüchter und Saatgutkaufleute Österreichs, Gumpenstein, pp. 51–55.
[3] Fernandez G. C. J. (1992) Stress tolerance index – a new indicator of tolerance. *HortScience*27, 626d-626.
[4] Chen X., Min D., Yasir T. A. and Hu Y. G. (2012) Evaluation of 14 morphological, yield related and physiological traits as indicators of drought tolerance in Chinese winter bread wheat revealed by analysis of the membership function value of drought tolerance (MFVD*). Field Crops Research* 137, 195–201.
[5] Blackmore T., Thomas I., McMahon R., Powell W. and Hegarty M. (2015) Genetic-geographic correlation revealed across a broad European ecotypic sample of perennial ryegrass (*Lolium perenne*) using array-based SNP genotyping. *Theoretical and Applied Genetics* 128, 1917–1932.
[6] RC Team (2013) R: A language and environment for statistical computing. https://cran.r-project.org/doc/manuals/r-release/fullrefman.pdf
[7] Karssen L. C., van Duijn C. and Aulchenko Y. (2016) The GenABEL project for statisticalgenomics'. *F1000Research* 5, 914.
[8] Breseghello F. and Sorrells M. E. (2006) Association mapping of kernel size and milling quality in wheat (*Triticum aestivum* L.) cultivars. *Genetics* 172, 1165–1177.

snpGBS: A Simple and Flexible Bioinformatics Workflow to Identify SNPs from Genotyping-by-Sequencing Data

Jie Kang[1,2,3], Ken Dodds[2], Stephen Byrne[3], Marty Faville[4], Michael Black[5], Andrew Hess[2], Melanie Hess[2], Alan McCulloch[2], Jeanne Jacobs[6], Dan Milbourne[3], Phillip Wilcox[1] and Rudiger Brauning[2]

[1] Department of Mathematics and Statistics, University of Otago, Dunedin, New Zealand
[2] AgResearch Invermay, Invermay Agricultural Centre, Mosgiel, New Zealand
[3] Teagasc, Oak Park, Crops Research Centre, Carlow, Ireland
[4] AgResearch Grasslands, Grasslands Research Centre, Palmerston North, New Zealand
[5] Department of Biochemistry, University of Otago, Dunedin, New Zealand
[6] AgResearch Lincoln, Lincoln Science Centre, Lincoln, New Zealand

Jie.kang@agreserach.co.nz

Abstract. Existing methods of SNP calling aim to optimise the efficiency by aggressively filtering and trimming GBS data. Conversely, we aim to capture more variation by following a mild workflow, which we have named snpGBS. Our results showed that including the extra variation yields a substantially larger number of SNPs from a ryegrass GBS dataset, which raised the question of whether this method is more appropriate for identifying SNPs from ryegrass GBS data.

Keywords: SNP, GBS, perennial ryegrass.

1 Background

Genotyping-by-Sequencing reduces genomic complexity by digesting DNA molecules into small fragments using restriction enzyme(s) [2]. This approach can be considered as a sampling process of genomic fragments, where the number and size of these fragments are determined by the choice of enzyme, polymorphic level of the targeted species and total sequencing effort.

The highly flexible nature of GBS makes it a particularly attractive method for genotyping perennial ryegrass (*Lolium perenne* L.), which is a highly diverse species with a relatively large genome (ca. 2.7 Gb). Such flexibility, on the other hand, makes the downstream bioinformatics processing more challenging due to the extra source of variation introduced by the sampling-based approach.

Several bioinformatics pipelines have been developed to identify single nucleotide polymorphisms (SNPs) from GBS data. Most involve filtering to enhance

accuracy, while some trim GBS reads to uniform-length tags to improve efficiency [5, 10]. An obvious drawback is the potential loss of information; especially when working on highly polymorphic species such as ryegrass. snpGBS, on the other hand, is a flexible workflow to identify SNPs from GBS data with minimal filtering.

2 Materials and Methods

2.1 GBS Data

Ninety-six samples were selected from a perennial ryegrass training population as described in [4], for which GBS had been performed using previously established methods [3]. Briefly, DNA was isolated from leaf tissue samples, then digested using the *Ape*KI restriction enzyme (NEB). Each GBS sample was ligated to a unique barcode identifier and a common adapter before merging into a 96-plex library. GBS libraries were each sequenced on two lanes of an Illumina HiSeq 2500 flowcell at AgResearch Invermay, New Zealand.

2.2 Bioinformatic Processing and Data Analyses

snpGBS involves demultiplexing raw GBS reads using cutadapt [10], mapping demultiplexed reads back to the same reference genome as described in [3] with bowtie2 [7], and finally, SNP calling using bcftools [8] with default options. For comparison, SNPs had been also identified using UNEAK [9] and TASSEL5 [4]. Genetic analyses of different SNP datasets were carried out using KGD [1]. Details can be found in the snpGBS repository (https://github.com/AgResearch/snpGBS).

3 Results and Discussion

Using snpGBS, we identified 1,305,406 SNPs with an average depth of 1.9 from the ryegrass GBS data. In comparison, 267,720 and 254,085 SNPs at similar depths were scored by UNEAK and TASSEL5 respectively. Note that both UNEAK and TASSEL5 were implemented with filtering based on minor allele frequency (MAF >= 0.03) and locus coverage (>= 0.1), whereas snpGBS only filters on mapping quality (MAPQ > 20). To make a fair comparison, we then explicitly filtered snpGBS output based on the same criteria. Interestingly, 830,207 SNPs were retained after the filtering, and 214,143 (26%) of which were identical to most SNPs (83%) identified by TASSEL5. This suggest that there may be a considerable amount of meaningful variation ignored by TASSEL5 (and UNEAK). One possible reason behind the differences in the number of

SNPs is that TASSEL5 (and UNEAK) trimmed GBS fragments into 64 bp tags, provided that the average number of SNPs per GBS fragment from snpGBS is much higher (Table 1).

We hypothesised that the extra information provided by snpGBS may benefit downstream analyses, such as relatedness estimation. However, the mean self-relatedness estimated from different SNP datasets in this case were very close. This may be caused by small sample size, and perhaps, in combination with low sequencing depth; we therefore aim to test our hypothesis using simulated GBS data generated by SimGBS [6]. With more SNPs being captured within each GBS fragment and minimal filtering being applied to maximise polymorphic level of potential short haplotypes (i.e., haplotypes that are constituted by SNPs located within each GBS fragment), we anticipate that snpGBS may be particularly useful for identifying SNPs to perform read-backed haplotyping using SMAP [11]. Because these short haplotypes are naturally a more powerful marker system, in terms of relatedness estimation; as well as for detecting strong linkage disequilibrium with casual causal variants, existing and new perennial ryegrass GBS data can be fully exploited to implement genomic selection [5].

Table 1. Summary Statistics of SNP Datasets Generated from Different Pipelines

	snpGBS	snpGBS-filtered	TASSEL5	UNEAK
Number of SNPs	1,305,406	830,207	254,079	267,720
Average Depth	1.92	2.35	2.57	1.02
Proportion of Missing Genotypes	0.48	0.39	0.28	0.56
Mean Self-Relatedness	1.02	1.01	1.00	1.04
Number of SNPs per GBS Fragment/Tag	4.56	3.75	2.37	1

References

[1] Dodds, Ken G., et al. (2015): Construction of relatedness matrices using genotyping-by-sequencing data. BMC Genomics 16.1 1–15.

[2] Elshire, Robert J., et al. (2011): A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PloS one 6.5: e19379.

[3] Faville, Marty J., et al. (2018): Predictive ability of genomic selection models in a multi-population perennial ryegrass training set using genotyping-by-sequencing. Theoretical and Applied Genetics 131.3: 703–720.

[4] Glaubitz, Jeffrey C., et al. (2014): TASSEL-GBS: a high capacity genotyping by sequencing analysis pipeline. PloS ONE 9.2: e90346.

[5] Hayes, Benjamin J., et al. (2013): Prospects for genomic selection in forage plant species." Plant Breeding 132.2: 133–143.

[6] Hess, Andrew S., et al. (2018): A method to simulate low-depth genotyping-by-sequencing data for testing genomic analyses. Proc 11th World Congr Genet Appl to Livest Prod 385.

[7] Langmead, Ben and Salzberg, Steven L (2012): Fast gapped-read alignment with Bowtie 2. Nature Methods 9.4 357.

[8] Li, Heng (2011): A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics 27.21: 2987–2993.
[9] Lu, Fei, et al. (2013): Switchgrass genomic diversity, ploidy, and evolution: novel insights from a network-based SNP discovery protocol." PLoS Genet 9.1: e1003215.
[10] Martin, Marcel (2011): Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet. journal 17.1: 10–12.
[11] Ruttink, Tom (2021): SMAP: a versatile approach to read-backed haplotyping in stacked NGS read data.” [Online]. Available online at https://gitlab.com/truttink/smap/

DOI: 10.5507/vup.21.24459677.17

Optimisation of GBS protocols for efficient genotyping of forage species

Bernadette Julier[1], Sébastien Blugeon[1], Sabrina Delaunay[1], Gaëtan Mappa[1], Tom Ruttink[2], Marie Pégard[1] and Philippe Barre[1]

[1] INRAE P3F, 86600 Lusignan, France
[2] ILVO Plant Science Unit, 9090 Melle, Belgium

bernadette.julier@inrae.fr

Abstract. GBS is an efficient genotyping tool for heterozygous and polyploid species, yet the fraction of missing data, inherent to the method, may be detrimental to genetic analyses. We tested several restriction enzymes to choose the ones that were likely to provide the maximum number of loci with little missing data considering a certain sequencing effort. For lucerne and cocksfoot, the enzymes that met the target of about 10 000 loci were the combination of *Pst*I-*Mse*I for lucerne and *Pst*I for cocksfoot. 1 066 lucerne accessions were genotyped with *Pst*I-*Mse*I, and more than 200 000 SNP with less than 5% missing data were obtained. Based on these results, we recommend performing such a test to optimize GBS genotyping efforts in forage species.

Keywords: cocksfoot, heterozygous, lucerne, polyploidy, SNP.

1 Introduction

GBS (Genotyping-by-Sequencing) previously showed to be a cost-efficient and reliable method to genotype several forage species [1] [2]. GBS is based on a reduction of genome complexity by the use of restriction enzymes that delimit genome portions (loci) which are amplified and sequenced [3]. In some cases, when the number of loci is high compared to the sequencing effort, genotyping matrices are generated with missing data for many loci. The optimization of GBS protocols is thus required to get the highest number of loci with the lowest fraction of missing data. We tested several restriction enzymes and evaluated the number of loci obtained in two species, lucerne (*Medicago sativa*) and cocksfoot (*Dactylis glomerata*). For lucerne, we also determined the number of SNP and missing data obtained on 1 066 accessions.

2 Material and Methods

For a lucerne individual genotype, 16 libraries were prepared with different restriction enzymes: *Ape*KI, *Pst*I, *Eco*RI-*Bam*HI, *Eco*RI-*Hind*III, *Eco*RI-*Mse*I, *Eco*RI-*Msp*I, *Eco*RI-*Ape*KI, *Pst*I-*Ape*KI, *Pst*I-*Mse*I, *Pst*I-*Msp*I, *Sbf*I-*Eco*RI, *Sbf*I-*Ape*KI, *Sbf*I-*Bam*HI, *Sbf*I-*Hind*III, *Sbf*I-*Mse*I, *Sbf*I-*Msp*I. Based on the distribution of the size of the fragments of each library, six were selected for sequencing (Fig. 1). The reads were mapped on a lucerne reference genome [4] and the number of loci that contained at least 10 reads was counted. Computational subsampling of data was performed to simulate varying numbers of reads per library. The number of loci was plotted as a function of library size. The objective was to select an enzyme or an enzyme pair that gave a saturation curve with a clear plateau of observed loci. From this curve, the number of loci at the plateau (here expected to be at least 10 000 loci) was used to determine the required number of reads per library. The same procedure was applied on cocksfoot with two single-enzymes and six enzyme pairs (Fig. 2). The reads were mapped on a draft reference genome sequence of cocksfoot (P. Barre & S. Buchman, unpubl.).

For lucerne, the most promising enzyme pair was used to genotype 1 066 accessions, each represented by a pool of 100 individuals. At this population level, the SNP were retained when the read depth reached at least 30.

3 Results

For lucerne (Fig. 1), the number of loci as a function of the number of forward reads showed a saturating curve shape for two enzyme pairs only (*Pst*I-*Ape*KI and *Pst*I-*Mse*I). With *Pst*I-*Mse*I, 12 600 loci (with at least 10 reads) were obtained with 10 million forward reads, which is economically affordable in a scientific

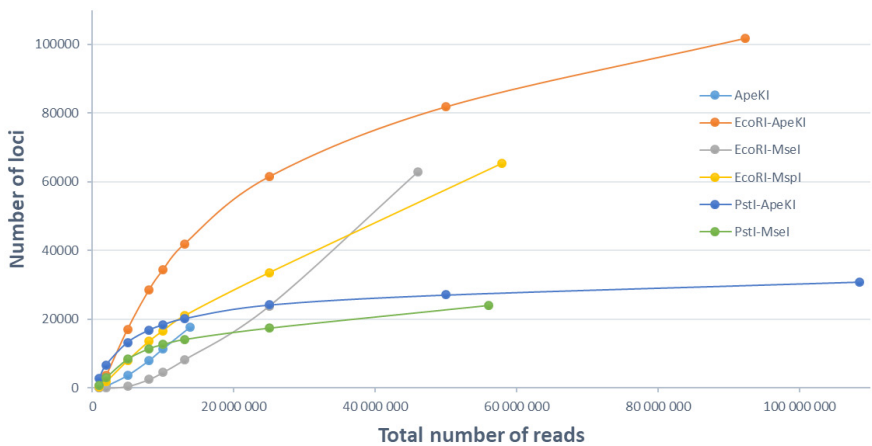


Fig. 1. Number of loci as a function of the number of reads obtained on one lucerne genotype, for *Ape*KI single-digest GBS and five combinations of two restriction enzymes.

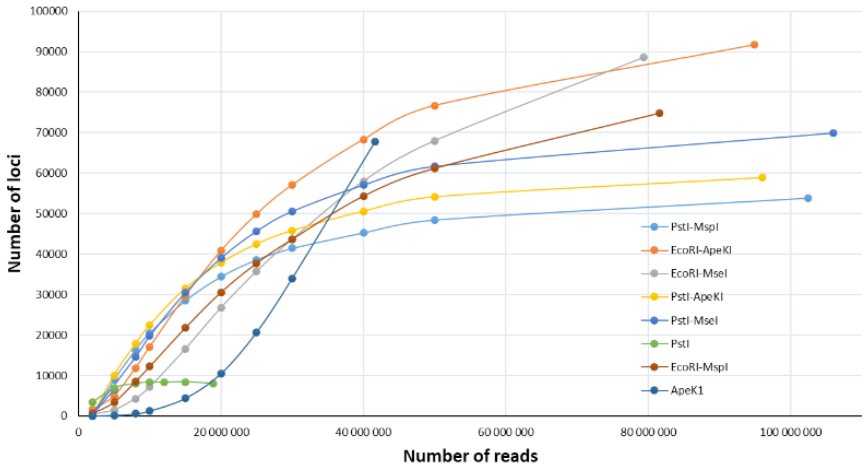


Fig. 2. Number of loci as a function of the number of reads obtained on one cocksfoot genotype, for *Pst*I, *Ape*KI single-digest GBS and six combinations of two restriction enzymes.

project. This pair of enzymes was chosen for GBS genotyping of 1 066 lucerne accessions. For cocksfoot, *Pst*I revealed almost 10 000 loci (with at least 10 reads) (Fig. 2) with 8 million total reads per individual.

For the 1 066 lucerne accessions, the mean number of forward reads obtained with *Pst*I-*Mse*I enzymes was 7.5 million. After trimming and filtering, reads were mapped on the reference genome [5], and we obtained 477 221 SNP for 1 061 accessions (5 accessions failed). With the objective to have less than 5% of missing data per SNP, a total of 228 568 SNP in 31 743 loci were generated. This dataset was sorted again to isolate a subset of SNP without missing data, and 118 421 SNP were then retained.

This study shows that a test of restriction enzymes is a way to optimize GBS protocols. The combination of *Pst*I-*Mse*I enzymes for lucerne and *Pst*I for cocksfoot were the most optimal to obtain a high number of markers with low proportion of missing data. A high-quality dataset comprising 228 568 SNP with less than 5% missing data on 1061 lucerne accessions has been obtained.

Acknowledgements

This study was supported by EUCLEG project (UE, H2020 n°727312) and Dactysec project (French Ministry of Agriculture Casdar n°C-2016-1).

References

[1] Julier, B., Barre, P., Lambroni, P., Delaunay, S., Thomasset, M., Lafaillette, F., Gensollen, V. (2018): Use of GBS markers to distinguish among lucerne varieties, with comparison to morphological traits. Mol. Breed. 38, 133.

[2] Byrne, S., Czaban, A., Studer, B., Panitz, F., Bendixen, C., Asp, T. (2013): Genome Wide Allele Frequency Fingerprints (GWAFFs) of populations via genotyping by sequencing. Plos One 8.

[3] Elshire, R.J., Glaubitz, J.C., Sun, Q., Poland, J.A., Kawamoto, K., Buckler, E.S., Mitchell, S.E. (2011): A Robust, Simple Genotyping-by-Sequencing (GBS) Approach for High Diversity Species. Plos One 6.

[4] Carrère, S., Gouzy, J., Debellé, F., Marande, W., Julier, B., Barre, P (2020): Medicago sativa cv. Mercedes genome sequence Available: https://hal.inrae.fr/hal-02993163v2/document.

[5] Chen, H.T., Zeng, Y., Yang, Y.Z., et al. (2020): Allele-aware chromosome-level genome assembly and efficient transgene-free genome editing for the autotetraploid cultivated alfalfa. Nature Communications 11.

DOI: 10.5507/vup.21.24459677.18

Elucidating the genetic control of southern anthracnose resistance in red clover

Lea A. Frey[1], Franz X. Schubiger[2], Tom Ruttink[3], Leif Skøt[4], Bruno Studer[1] and Roland Kölliker[1]

[1] Molecular Plant Breeding, Institute of Agricultural Sciences ETH Zurich, Universitaetstrasse 2, 8092 Zurich, Switzerland
[2] Fodder Crop Breeding, Agroscope Reckenholz, Reckenholzstrasse 191, 8046 Zurich, Switzerland
[3] Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Plant Sciences Unit, Caritasstraat 39, 9090 Melle, Belgium
[4] Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth University, Gogerddan, Aberystwyth, Wales, SY23 3EE, United Kingdom

lea.frey@usys.ethz.ch

Abstract. Red clover (Trifolium pratense L.) is an important forage legume of temperate regions, particularly valued for its high yield potential and its high forage quality. A worldwide collection of 397 red clover accessions was genotyped using a population-based genotyping-by-sequencing approach. Resistance to southern anthracnose, caused by Colletotrichum trifolii, was assessed in the greenhouse using spray inoculation. Mean plant survival rate for single-spore isolate inoculation was 23% across all accessions. Only very few accessions showed considerable resistance (survival rates > 50%), most of the accessions were highly susceptible. This highlights the urgent need to improve resistance to southern anthracnose in red clover. Genome-wide association analysis revealed several loci, which were significantly associated with the trait and may represent promising candidate genes for anthracnose resistance.

Keywords: Resistance, Trifolium pratense L., Colletotrichum trifolii, Genome-wide association study

1 Introduction

Red clover (*Trifolium pratense* L.) is one of the most important forage legumes in temperate climates, grown throughout Europe and North America. Red clover (2n=2x=14) is an outcrossing crop with a high degree of self-incompatibility and consequently a high intra-population genetic variability. Main breeding objectives are yield, quality, persistence, and disease resistance.

Colletotrichum trifolii Bain & Essary is a key fungal pathogen threatening red clover, it causes southern anthracnose that is characterised by brown lesions, chlorosis and wilting of the entire plant. As red clover is highly susceptible to southern anthracnose, valuable amounts of forage yield are lost every year. Fungal spores can be spread by rain and wind, particularly if conditions are warm and moist.

Recently, *C. trifolii* has benefitted from rising temperatures, and southern anthracnose became a limiting factor for red clover production, leading to an increased demand for resistant cultivars [1]. The present work aimed at investigating the genetic diversity in a diverse red clover panel and to identify loci associated with southern anthracnose resistance.

2 Materials and Methods

Red clover accessions (n = 397) from 28 different countries were grown in the greenhouse in a resolvable row-column design with four replications and two standard cultivars as checks. Spray inoculation was adapted from Schubiger et al. 2003 [6], using a single-spore isolate collected in Switzerland. For each accession, the survival rate was assessed on 24 plants per accession. Best linear unbiased predictors (BLUPs) were calculated using the ASReml library [2] of R (version 3.6.0).

All accessions were genotyped by LGC Genomics (Teddington, UK) using a genotyping-by-sequencing (GBS) approach with 200 pooled plants per accession. Single nucleotide polymorphisms (SNP) were identified using SNAPE-pooled [5]. In total 30,668 SNPs were selected (read depth > 30 in > 95% of accessions) and a minor allele frequency threshold of 5% in at least ten accessions was set. Kinship matrix was constructed as described in Cericola et al. 2018 [3], using only SNP with no missing data (12,272 SNPs). Associations were calculated with the Circulating Probability Unification (FarmCPU) method implemented in the GAPIT R package.

3 Results and Discussion

Only ten accession showed a substantial resistance to *C. trifolii* (survival rate > 60%), most of the accessions were highly susceptible (mean survival rate 23%; range 0%–80%). Principle component analysis based on GBS data detected genetic variation among the 397 accessions. Genome-wide association analysis revealed over ten SNPs that were significantly associated with southern anthracnose resistance (Fig. 1).

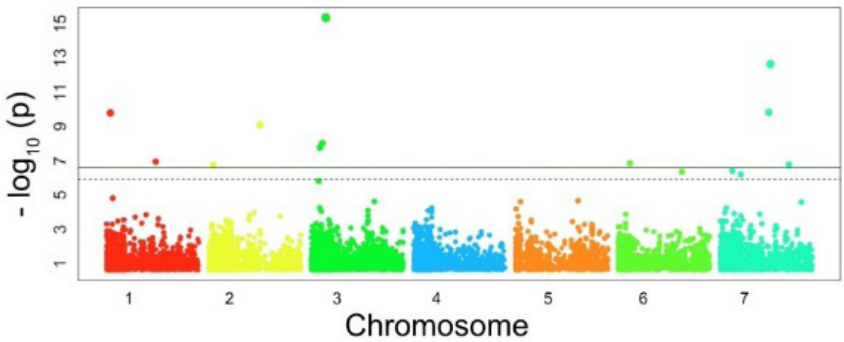


Fig. 1. Manhattan plot showing candidate SNPs and p-values from GWAS using FarmCPU. The solid line is the significance threshold after Bonferroni correction with an α of 5%, and the dashed line represents the threshold from the FDR.

While the genetic control of southern anthracnose resistance in red clover is largely unknown, resistance to *Colletotrichum* spp. has been studied in other plant species including soybean (*Glycine max* L.), common bean (*Phaseolus vulgaris* L.) and alfalfa (*Medicago sativa* L.) [4]. In alfalfa, two dominant resistance genes (*An1* and *An2*) are known, which explain most of the resistance to the three known races of *C. trifolii* [7].

4 Conclusion

Most of the tested red clover accessions were highly susceptible to *C. trifolii*. This highlights the urgent need to improve resistance to southern anthracnose in current breeding programs. Nevertheless, a few accessions were resistant and can be used as a resistance source for resistance breeding. Associated SNPs will be further validated and present the first step in finding underlying genes responsible for southern anthracnose resistance in red clover.

Acknowledgements

This work was financially supported by the EU's Horizon 2020 Programme for Research & Innovation (grant agreement n°727312; EUCLEG).

References

[1] Boller B, Schubiger FX, Kölliker R (2010): Red Clover. In: Boller B, Posselt UK, Veronesi F (eds) Fodder Crops and Amenity Grasses, Springer New York, New York, pp. 439–455.
[2] Butler D (2020) asreml: Fits the Linear Mixed Model. R package version 4.1.0.143. www.vsni.co.uk

[3] Cericola F, Lenk I, Fè D, Byrne S, Jensen CS, Pedersen MG, Asp T, Jensen J, Janss L (2018): Optimized Use of Low-Depth Genotyping-by-Sequencing for Genomic Prediction Among Multi-Parental Family Pools and Single Plants in Perennial Ryegrass (Lolium perenne L.). Front Plant Sci 9:369.
[4] Dean R, Van Kan JAL, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J, Foster GD (2012): The Top 10 fungal pathogens in molecular plant pathology. Mol Plant Pathol 13(4):414–430.
[5] Raineri E, Ferretti L, Esteve-Codina A, Nevado B, Heath S, Perez-Enciso M (2012): SNP calling by sequencing pooled samples. BMC bioinformatics 13(1):239.
[6] Schubiger FX, Streckeisen P, Boller B (2003) Resistance to Southern Anthracnose (Colletotrichum trifolii) in Cultivars of Red Clover (Trifolium pratense). Czech J Genet Plant Breed 39:309–312
[7] Yang S, Gao M, Xu C, Gao J, Deshpande S, Lin S, Roe BA, Zhu H (2008) Alfalfa benefits from Medicago truncatula: The RCT1 gene from M. truncatula confers broad-spectrum resistance to anthracnose in alfalfa. Proc Natl Acad Sci 105(34):12164–12169.

DOI: 10.5507/vup.21.24459677.19

Genotyping-by-sequencing (GBS) of white clover (*Trifolium repens*) F_1 families as pools

Katie Hetherington[1,2], Leif Skot[2], Dan Milbourne[1] and Stephen Byrne[1]

[1] Teagasc, Crop Science Department, Oak Park, Carlow, R93 XE12, Ireland
[2] Institute of Biological, Environmental and Rural Science, Aberystwyth University, Aberystwyth, Ceredigion, United Kingdom.

katie.hetherington@teagasc.ie

Abstract. GBS of F_1 families may offer an opportunity to exploit historical data in white clover breeding programmes where seed of F_1 families exists in long term storage. Here, we used a GBS strategy to genotype individuals and replicate family pools. We compared allele frequencies calculated from genotyping of individuals within a family to those calculated from genotyping of pools of individuals within a family. Allele frequency profiles had high reproducibility at moderate read depths across replicate pools, and allele frequencies in pools were comparable to allele frequencies determined from individual plant genotyping.

Keywords: white clover, genotyping, GBS.

1 Background

White clover can contribute to both the economic and environmental sustainability of pastoral based production systems. As white clover is predominantly grown in mixtures with perennial ryegrass it needs to be present in a sufficient quantity to take advantage of its higher nutritive quality and nitrogen-fixing ability. Opportunities exist to accelerate genetic gain in white clover breeding using technologies such as genomic selection. A prerequisite to developing genomic selection is a training population where phenotypes and genotypes have been collected. Ideally, genotyping parents of full-sib or half-sib families would be possible; however in many cases the parental material is not available for families that have been evaluated in the past. In order to exploit historical data in breeding programmes we need to genotype family pools for which seed exist in long term storage; a strategy already employed in perennial ryegrass F_2 families [1]. Here, we established an experiment to genotype F1 family pools (offspring of a cross between two heterozygous individuals) of white clover (allotetraploid) to evaluate reproducibility of sampling approach and accuracy of allele frequencies.

2 Methods

A single F1 family was selected for use in this experiment and seed was germinated in seedling trays and sampled for DNA isolation when sufficient growth was achieved. In the first case leaf samples were taken from 48 individual seedlings and for genotyping as individual plants. In the second case, a pool consisting of a single leaf from each of 48 plants was constructed (this was repeated three times, each time taking a new leaf, to create three replicate pools). DNA was isolated from the 48 individual samples and three pools and GBS was carried out by LGC Genomics (Berlin) using the restriction enzyme *Ms*II for genome complexity reduction (as selected after the results from a pilot study by LGC). The libraries were sequenced on an Illumina NextSeq as 150 bp paired-end reads. The read pairs were aligned back to the reference genome [2] using bwa [3], and bcftools [4] was used with a minimum mapping quality of 30 to generate genotype calls and report allele read depths. VCFtools [5] was then used to filter putative variants and generate a final variant set (--minDP 6 , --maf 0.10 --max-maf 0.90 --minGQ 20 --max-missing 0.20). We first determined the frequency of the non-reference allele at each variant site in the three replicate pools (we use AAF_{pool} to denote Alternative Allele Frequency in pools, to be consistent with terminology in [6]). When the total read depth was at least 30, the AAF_{pool} was calculated, otherwise a missing value was reported. In the case of the individuals, genotype calls were determined when the genotype quality was at least 30 and the AAF for the F_1 family was determined from the 48 individuals (denoted as AAF_{ind}) when there were eight or less individuals with missing data (we first called genotypes calls and then used these to calculate AAF). Variant sites without missing data in AAF_{ind} and the three AAF_{pool} were then used to determine Pearson correlations between pools and between pools and the AAF determined from individuals.

3 Results and Discussion

An average of 2.4 million read pairs (after filtering) were generated for each of the 48 individuals and three F_1 family pools. The reads from the GBS with *Ms*II aligned were well distributed across all homologous chromosomes, and 140,000 putative SNPs were identified within the F1 family. After determining AAF_{ind} and AAF_{pool}, a subset of 16,504 SNPs with full data were used to compare AAF between the three pools and the AAF calculated from individuals, and also to compare the AAF between the three pools (Figure 1). The AAF from the 48 individuals had a tendency to cluster around 0.25, with some clustering around 0.50, and 0.75 which is not unexpected for AAF in an F_1 family. The Pearson correlation between the AAF calculated in the three pools and the AAF calculated from the 48 individuals was high (0.84 – 0.88) indicating that the pooling strategy does a good job of estimating the true AAF. The Pearson correlation between the three pool replicates was also high (0.86 – 0.87) indicating that the

sampling approach is reproducible. Improvements in correlation can be achieved by increasing the read depth thresholds beyond 30 but at the expense of increasing missing data or requiring an increased sequencing budget. Our results indicate that GBS of pooled samples is a feasible strategy to determine the AAF in F_1 families and opens the possibility of developing genomic selection from historical white clover breeding data.

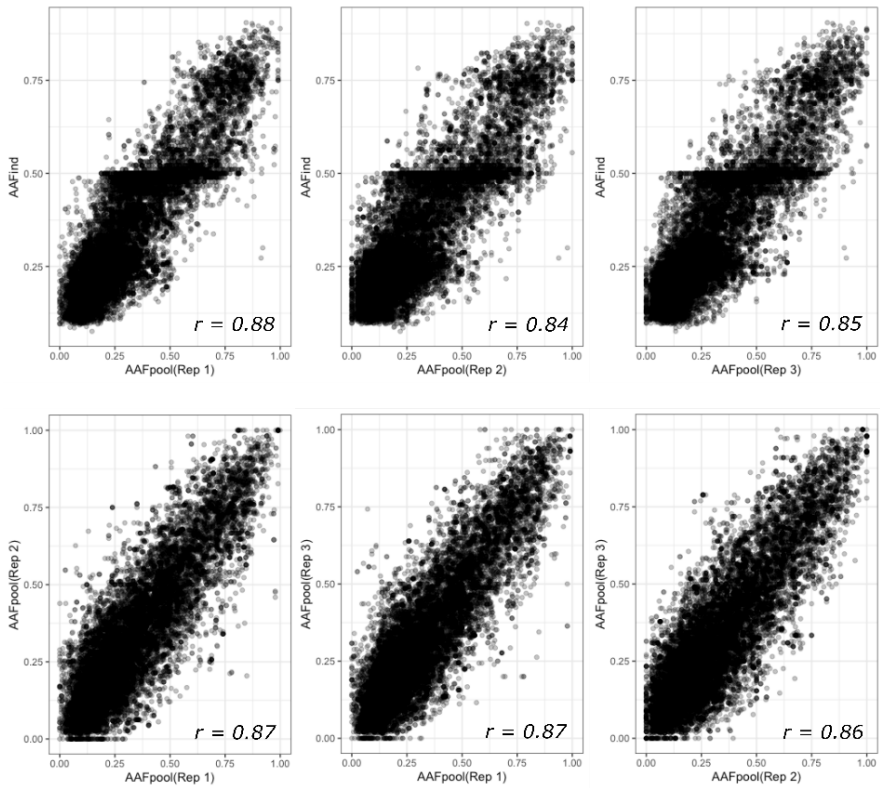


Fig. 1. Scatter plots and Pearson correlations between Alternative Allele Frequency (AAF) determined through genotyping of individuals and pools (top) and between replicate pools (bottom).

References

[1] Fè, D., Ashraf, B.H., Pedersen, M.G., Janss, L., Byrne, S., Roulund, N., Lenk, I., Didion, T., Asp, T., Jensen, C.S. and Jensen, J. (2016): Accuracy of genomic prediction in a commercial perennial ryegrass breeding program. The Plant Genome, 9(3).

[2] Griffiths, A.G., Moraga, R., Tausen, M., Gupta, V., Bilton, T.P., Campbell, M.A., Ashby, R., et al.(2019): Breaking free: The genomics of allopolyploidy-facilitated niche expansion in white clover. The Plant Cell 31.

[3] Li, H. and Durbin, R. (2010): Fast and accurate long-read alignment with Burrows–Wheeler transform. Bioinformatics, 26(5).

[4] Danecek, P., Bonfield, J.K., Liddle, J., Marshall, J., Ohan, V., Pollard, M.O., Whitwham, A., Keane, T., McCarthy, S.A., Davies, R.M. and Li, H., (2021): Twelve years of SAMtools and BCFtools. Gigascience, 10(2), p.giab008.

[5] Danecek, P., Auton, A., Abecasis, G., Albers, C.A., Banks, E., DePristo, M.A., Handsaker, R.E., Lunter, G., Marth, G.T., Sherry, S.T. and McVean, G., (2011): The variant call format and VCFtools. Bioinformatics, 27(15), pp.2156–2158.

[6] Verwimp, C., Ruttink, T., Muylle, H., Van Glabeke, S., Cnops, G., Quataert, P., Honnay, O. and Roldan-Ruiz, I., (2018): Temporal changes in genetic diversity and forage yield of perennial ryegrass in monoculture and in combination with red clover in swards. PloS one, 13(11), p.e0206571.

DOI: 10.5507/vup.21.24459677.20

Using marker assisted parental analysis to move to full sib selection in breeding perennial ryegrass

An Ghesquiere, Hilde Muylle and Joost Baert

Flanders research institute for Agriculture, Fisheries and Food (ILVO), Caritasstraat 39, 9090 Melle, Belgium

an.ghesquiere@ilvo.vlaanderen.be

Abstract. The aim of this study was to evaluate the use of molecular marker parental analysis in the construction of polycrosses to enhance the yield in a *Lolium perenne* breeding programme. In current practice there is no selection of the male contribution in the construction of synthetics. By using molecular markers we performed full sib selection instead of half sib selection. A polycross consisting of 18 components was used as starting material. After identification of the four highest yielding halfsibs, four different approaches to construct the second generation synthetic were compared. Two approaches used seedlings from the remnant seed of the four halfsib families, one with paternal selection based on molecular markers, choosing only seedlings that are a cross between the maternal parents of the 4 highest yielding halfsibs and one without paternal selection. The other two were established with shoots from the halfsib yield trial, one with and one without paternal selection. The progenies of the four polycrosses were evaluated in a yield trial. There were no significant differences, but the progenies based on parental selection tended to be higher yielding.

Keywords: *Lolium* perenne, Parental Analysis, Breeding, Molecular Marker, SSR

1 Introduction

Perennial ryegrass is a wind-pollinated species and polycross progenies are produced through random mating of the selected parental plants. Each maternal plant is pollinated by all the other parental plants. Testing the halfsibs reveals that some of them have a lower agronomic performance. For the construction of the second generation of the synthetics, plants from only the best performing halfsib families are intercrossed. In current practice there is no selection of the male contribution in the construction of the second generation of the synthetics. In this study we used molecular markers to identify those progeny plants that are the result of a cross between the maternal parents of the best performing halfsib families, i.e. full sib selection. We constructed polycrosses with these selected plants and compared the progenies with progenies of the polycrosses set up without paternal selection.

2 Material and Methods

In 2011 we planted a polycross of 18 parents. Halfsib seeds were harvested on each parental genotype in July 2012. For determination of the yield of the halfsibs, a plot (8 m^2) trial was established in May 2013 in a randomized block design with 3 replicates. The trial was cut 4 times in 2014 and 4 times in 2015 and the dry matter yield was measured. Based on the results, we selected the 4 highest yielding halfsibs.

The second generation synthetic was constructed in 4 different approaches: for the first two multiplications we used remnant seeds of the four selected halfsibs: we sowed 588 seeds of each of the four selected halfsibs and used part of the seedlings for a multiplication without paternal analysis (RS), and part of the seedlings for a multiplication after paternal analysis using only the seedlings of which both parents had high yielding progenies (PRS). For the last two multiplications we took 588 shoots in the yield trial of the halfsibs, and used part of the shoots for a multiplication without paternal analysis (SH), and part of the shoots for a multiplication after paternal analysis using only the shoots of which both parents had high yielding progenies (PSH).

For the parental analysis 490 seedlings and 490 shoots were analysed using SSRs as described in Studer et al. (2010) [1]. Parental analysis was performed using Cervus 3.0 [2].

In each of the four multiplications, 28 plants per halfsib were selected according to the approach taken (RS, PRS, SH and PSH) and planted in a polycross scheme and seeds were harvested in 2016. The synthetics obtained in the 4 approaches (RS, PRS, SH and PSH) were tested in a yield trial that was established in April 2018 in a randomized block design with 3 replicates. The trial was mown 4 times in 2019 and 4 times in 2020. The ANOVA analysis was performed using Statistica® version 13.5.0.17.

3 Results and Discussion

Figure 1 shows the dry matter yield of the second generation synthetics produced in the 4 different approaches (RS: remnant seed, PRS: remnant seed and paternal selection, SH : shoots, PSH: shoots after paternal selection). There are no significant differences but only a trend in both testing years that integration of marker assisted parental analysis results in higher yield. Starting from remnant seed without parental analyses seems to results in a lower production.

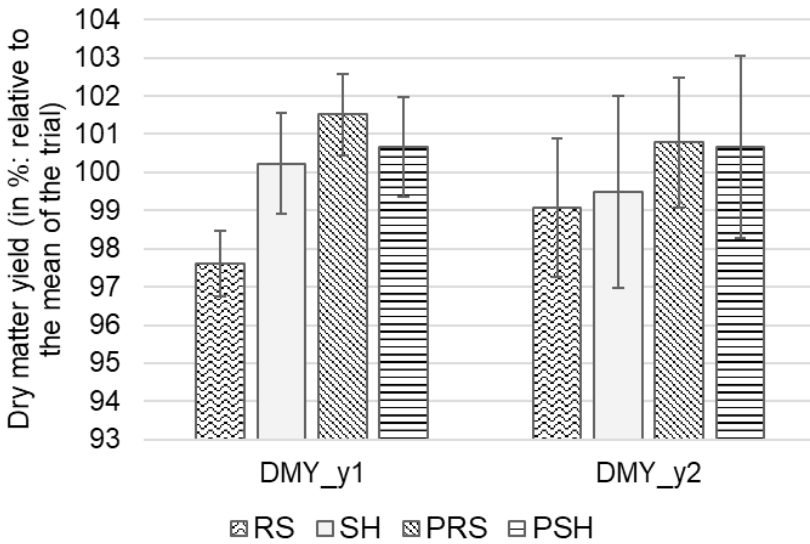


Fig. 1. Total dry matter yield in 2019 and 2020 (relative to the mean of the total DMY) of the second generation synthetics based on 4 different multiplication approaches RS: synthetic established with seedlings without parental analysis; SH: synthetic established with shoots from the selected halfsib plots without parental analysis; PRS: synthetic established with seedlings selected for a high yielding male parent; PSH: synthetic established with shoots from the selected halfsib plots and selected for a high yielding male parent.

4 Conclusion

The total dry matter yield of the synthetics based on parental selection tends to be higher than of the synthetics without parental selection. Integration of marker assisted parentage analysis to identify the progeny plants that are the results of a cross between two maternal parents of high yielding halfsib families could result in higher yielding synthetics. Further research is needed to confirm this.

References

[1] Studer, B., Kölliker, R., Muylle, H. et al. (2010): EST-derived SSR markers used as anchor loci for the construction of a consensus linkage map in ryegrass (Lolium spp.). BMC Plant Biol 10,177.

[2] Kalinowski, ST, Taper, ML, Marshall, TC (2007): Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Molecular Ecology 16:1099–1106.

 DOI: 10.5507/vup.21.24459677.21

Assessing photoperiod regulation of early development in perennial ryegrass varieties grown *in vitro*

Michael Richards[1], David Dalton[1]
and Rossana Henriques[1,2] [0000-0002-9394-0098]

[1] School of Biological, Earth and Environmental Sciences, University College Cork, Distillery Fields, North Mall, Cork T23 TK30, Ireland
[2] Environmental Research Institute, University College Cork, Lee Road, Cork, Ireland

rossana.henriques@ucc.ie

Abstract. Plants utilise light signals to determine their location, time of the day and neighbour proximity. In addition, light acts as an input to the circadian clock. This timekeeping mechanism is necessary for seasonal perception, ensuring the coordination between growth and development with environmental conditions. Perennial ryegrass (*Lolium perenne*) is the major forage crop in Ireland and its growth season is affected both by photoperiod and temperature changes. However, assessing growth responses under field conditions is laborious and time consuming, especially at the early stages of breeding. Here, we describe a simple, non-expensive and fast *in-vitro* screening method to assess growth parameters at early developmental stages under different photoperiod conditions. Our analysis of 5-day old seedlings identified differences between photoperiods (short and long days) and varieties in terms of root and shoot length, suggesting it could provide an initial read-out of growth responses preceding field tests. Moreover, since this method can incorporate different photoperiod and temperature conditions, it would be able to inform on early developmental responses of new perennial ryegrass varieties grown under current and future climate conditions, which would be useful for breeding programs.

Keywords: perennial ryegrass, photoperiod, circadian clock.

1 Introduction

Plants use light and temperature cues to determine their surroundings, neighbours, time of the day and season. This perception relies on the combined action of photoreceptor signalling cascades and the circadian clock [1–3]. Due to its transcriptional and translational networks, the clock generates robust biological rhythms allowing plants to anticipate and predict daily and seasonal events. This ability to coordinate internal and external cues increases plant fitness and biomass, due to regulation of leaf growth, photosynthesis, sugar metabolism, flowering time and abiotic stress responses [3]. Perennial ryegrass is Ireland's

main forage crop, covering over 80% of its agricultural land; and its growth follows a seasonal pattern, peaking from late spring to early summer and slowly declining, being the lowest at the winter months [4] [5]. Currently, breeding programs assess variety performance in the field, which is time-consuming and laborious. Therefore, new methodologies providing faster initial screens would be beneficial. Here, we report a simple method to assess root and shoot length in perennial ryegrass seedlings as early as 5 days old. Moreover, our protocol can be implemented under different light and temperature settings; allowing to test current and future climate conditions, something that is not feasible in the field.

2 Material and Methods

Seeds from perennial ryegrass diploid varieties (Astonconqueror, Gusto, Kerry, Nifty and Oakpark) supplied by Teagasc and Goldcrop™ were surface sterilized with 70% ethanol, incubated in a 50% bleach solution in 0.05% Triton-X100, washed with sterile distilled water and placed in square plates of 1xMS medium lacking both Nitrogen and sucrose. After 1 week of incubation at 4°C in the dark, plates were placed vertically in a growth chamber under either short days (SD, 8h light/16h dark) or long days (LD, 16h light/8h dark) conditions (white light 100 $\mu molm^{-2}s^{-1}$, 22°C). Root and shoot length were recorded once daily from day 3 to 7 in light, and day 5 selected as optimal for further analyses. ImageJ (https://imagej.nih.gov/ij/) was used for measurements and statistical analysis was performed with Prism 7 (GraphPad) software.

3 Results and Discussion

3.1 Assessing shoot and root length under different photoperiods

After 5 days of light exposure, we observed that SD-grown seedlings displayed shorter shoots and roots, but we did not observe any statistically significant difference among varieties (Fig. 1). Under LDs, only Nifty developed shorter roots and shoots, whereas all the other varieties had similar growth responses. These results show that our approach discriminates growth responses between different photoperiods/varieties. These seem to be more pronounced in longer days that are typical of the warmer season when perennial ryegrass growth is maximal [5].

3.2 Evaluation of root/shoot ratios under different photoperiods

Analysis of root/shoot ratios revealed clear differences between photoperiods and varieties. Whereas under LDs, shoot development seemed preferred; under SDs we observed a shift towards longer roots. In addition, root/shoot ratios in

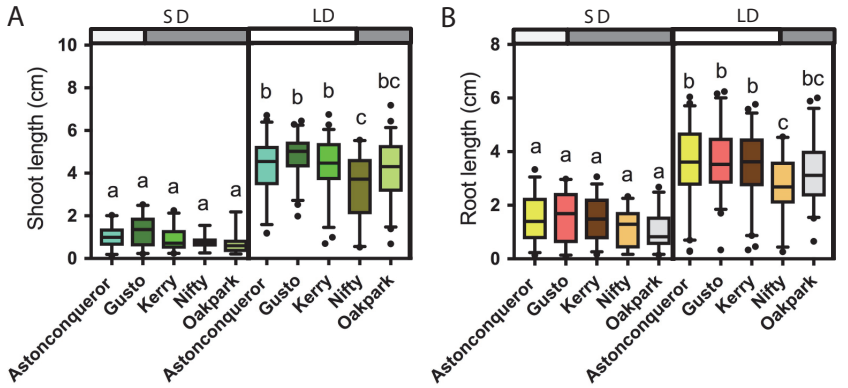


Fig. 1. Photoperiod regulation of growth responses in perennial ryegrass varieties. Shoot (**A**) and root (**B**) length were determined after 5 days in the light in two (SD) or three (LD) independent replicates (n=25 to 35 seedlings). Boxplot centre lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers delimit the 5th to 95th percentiles, outliers are represented by dots. Statistically significant differences were determined by One-Way ANOVA followed by Tukey's test. White and grey rectangles correspond to the light and dark periods, respectively.

the latter conditions were statistically different among four out of the five varieties tested (Table 1).

Table 1. Root/shoot ratios in perennial ryegrass seedlings grown under SD or LD conditions. Statistically significant differences determined by Two-Way ANOVA followed by Tukey's test.

Variety	Root/shoot ratio SD (mean±SEM)	Root/shoot ratio LD (mean±SEM)
Astonconqueror	1.45 ± 0.31 (a)	0.83 ± 0.09 (e)
Gusto	1.29 ± 0.13 (b)	0.76 ± 0.08 (e)
Kerry	1.67 ± 0.17 (c)	0.77 ± 0.05 (e)
Nifty	1.88 ± 0.01 (d)	0.82 ± 0.04 (e)
Oakpark	1.45 ± 0.17 (a)	0.8 ± 0.07 (e)

4 Conclusions

Our results identified differences in root and shoot length of varieties grown under distinct photoperiods. Therefore, we propose that this protocol could be used as an initial screening method in breeding programs. Besides photoperiod, this method can assess the role of temperature on root and shoot growth, making it possible to test how current and future climate conditions affect the early development of perennial ryegrass varieties. Further physiological, biochemical and molecular analyses could help identify the regulators providing growth advantages in a changing environment.

References

[1] Troein, C., Locke, J. C., Turner, M. S., Millar, A. J. (2009): Weather and seasons together demand complex biological clocks. Curr Biol 19, 1961–1964.
[2] Greenham K. et al. (2017): Geographic variation of plant circadian clock function in natural and agricultural settings. Journal of Biological Rhythms 32, 26–34.
[3] Dodd A. et al. (2005): Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. Science 309, 630–633.
[4] Humphreys, M. W., et al. (2006): A changing climate for grassland research. The New phytologist 169, 9–26.
[5] Förster, L., et al. (2018): Growth under cold conditions in a wide perennial ryegrass panel is under tight physiological control. PeerJ 6, e5520.

Session 3: Strategies to optimally exploit genetic diversity

 DOI: 10.5507/vup.21.24459677.22

Can we define a persistent red clover ideotype for mixtures with grasses?

Åshild Ergon[1]

[1] Norwegian University of Life Sciences, Faculty of Biosciences, Ås, Norway

ashild.ergon@nmbu.no

Abstract. Taking the conditions in species mixtures into consideration when breeding forage species is challenging but could result in better varieties. We found that offspring of red clover survivors from mixtures had earlier petiole and stem elongation than offspring of survivors from pure stands, while those from pure stands had better freezing tolerance. Petiole length and timing of stem elongation could easily be scored in a large number of plants under controlled conditions and used in further crossing, possibly helping to improve persistence of red clover to be cultivated in mixtures with grasses. Careful monitoring of possible trade-offs with yield, winter survival and forage quality would be necessary.

Keywords: breeding, Nordic, survivor populations, *Trifolium pratense*

1 Introduction

The advantages of cultivating grass-legume mixtures are well-established [1]. It is also known that the agronomic performance of a genotype is influenced by the surrounding plant community [2]. In spite of this, forage breeding is for various reasons almost exclusively based on performance in pure stands. Litrico and Violle [2] and Annicchiarico et al. [3] have argued why and how selection aimed at improved performance of mixtures could be achieved.

In the Nordic region red clover (*Trifolium pratense*) is mainly cultivated in mixtures with grasses intended for mechanical harvesting, sometimes combined with grazing. In such systems, red clover is less persistent than its companion species, and improving persistence is therefore a breeding goal. Persistence is a complex trait, dependent on both the abiotic and biotic environment. Limited persistence of legumes relative to their grass companions is frequently assigned to an inferior competitive ability under moderate to high N fertilization levels. In a Nordic context, red clover can be rather dominant in the second year and winter survival is an important component of persistence.

2 Red Clover Persistence in Mixtures in a Nordic Climate

In an experiment in south-eastern Norway [4] with limited N fertilization, we studied the year-to-year species dynamics of mixtures containing red and white clover (*T. repens*), perennial ryegrass (*Lolium perenne*) and tall fescue (*Festuca arundinacea*), based on annual yields of each species in the mixture. We found that from year 1 (first production year) to year 2 in a normal 3 cut system, red clover was slightly inferior to tall fescue, but dominant to perennial ryegrass and white clover. From year 2 to year 3, however, red clover was inferior to all the other species, but this appeared to be largely due to the high mortality of red clover during the previous winter, where conditions were quite severe. Interestingly, red clover winter mortality was higher in pure stands than in mixtures, but it is not known if this was due to conditions during winter or during the previous growing season.

Already before this winter survivors from pure stands and mixtures had differentiated genetically, as determined by analysis of GBS-derived SNP allele frequencies [5]. For some chromosomal regions, different alleles had been selected in mixed vs. pure stands (false discovery rate < 0.05). In the autumn of year 3, survivor populations from mixtures and pure stands were collected, and the same was done in another experiment in central Norway. Offspring were generated from all survivor populations and characterized in experiments under controlled conditions [6]. Non-vernalized offspring of mixture survivors had earlier petiole elongation and earlier stem elongation, larger shoots (also when accounting for earliness) than offspring from pure stands had. Offspring of mixture survivors also had lower freezing tolerance of young cold acclimated plants. Better survival of genotypes with rapid petiole elongation in mixtures with grasses was also noted by others [3] and could possibly provide the ability to compete for light during establishment and regrowth when growing next to grasses. Similarly, genotypes with early stem elongation could be expected to compete better with grasses, which enter reproductive development earlier than red clover. Larger shoot size of offspring from mixtures indicates that also this trait provides a selective advantage for red clover in mixtures. This is in line with the positive correlation between shoot biomass and persistence of red clover varieties cultivated in mixtures with grasses found by Hoekstra et al. [7]. They also observed a correlation between persistence and plant height in mixtures. Plant height in red clover is linked to the timing of stem elongation, and in our experiment offspring of mixture survivors were not significantly taller when we corrected for earliness, although at the south-eastern location they did have longer average internode length than the originally sown population. Possible explanations for the lower freezing tolerance of offspring from mixture populations are less obvious or complex.

3 Could Persistent Ideotypes Be Useful for Breeding?

A range of options for incorporating mixture performance specifically in breeding goals, which I will not go into, are given by Annicchiarico et al. [3] and Litrico and Violle [2]. I will just consider the ideotype approach, which can complement breeding programs by helping to prioritize among traits, as well as generate testable hypotheses about how changes in one trait could affect the overall performance of the plant or crop community [8]. As more information is gained, an ideotype model describing the relationship between traits and performance in mixtures could be improved. Based on the limited information above a hypothetical ideotype for persistent red clover to be cultivated in mixtures would have early petiole and stem elongation and large shoot size. First of all, the hypothesis that these traits provide better persistence in mixtures relative to pure stands would have to be tested for relevant breeding material. Persistence is already a major selection trait in most breeding programs, but in pure stands. Applying additional selection for traits that are particularly important for persistence in mixed stands could possibly help achieving breeding goals faster. Early petiole and stem elongation could be easily scored in a large number of individuals under controlled conditions and selected individuals could be used for crossing. However, it would be necessary to carefully monitor possible trade-off effects on yield, winter survival and forage quality. In a recent study of a collection of Nordic red clover accessions (mainly wild populations and landraces), traits related to petiole length and shoot growth in general was to some extent negatively correlated with winter survival and freezing tolerance [9]. Moreover, earlier stem elongation is associated with a reduction in protein content and digestibility [10], a possible trade-off which should also be taken into account.

Acknowledgements

This study was funded by EU Grant Agreement No. FP7-244983 (Multisward) and Norwegian Research Council Project No. 225330 (Agropro).

References

[1] Lüscher A., Mueller-Harvey, Soussana J. F., Rees R. M. and Peyraud J. L. (2014): Potential of legume-based grassland-livestock systems in Europe: a review. Grass and Forage Science 69, 206–228.

[2] Litrico I. and Violle, C. (2015): Diversity in Plant Breeding: A New Conceptual Framework. Trends in Plant Science 20, 604–613.

[3] Annicchiarico P., Collins R. P., De Ron A. M., Firmat C., Litrico I. and Hauggaard-Nielsen H. (2019): Do we need specific breeding for legume-based mixtures? Advances in Agronomy 157, 141–215.

[4] Ergon Å., Kirwan L., Bleken M. A., Skjelvåg A. O. Collins R. P. and Rognli O.A. (2016): Species interactions in a grassland mixture under low nitrogen fertilization and two cutting frequencies: I. dry matter yield and dynamics of species composition. Grass and Forage Science 71, 667–682.

[5] Ergon Å., Skøt L., Sæther V. E. and Rognli O. A. (2019): Allele frequency changes provide evidence for selection and identification of candidate loci for survival in red clover (Trifolium pratense L.). Frontiers in Plant Science 10, 718.
[6] Ergon Å. and Bakken A. K. (2016): Red clover traits under selection in mixtures with grasses versus pure stands. Grassland Science in Europe 21, 811–813.
[7] Hoekstra N.J., De Deyn G. B., Xu Y., Prinsen R. and Van Eekeren N. (2018): Red clover varieties of Mattenklee type have higher production, protein yield and persistence than Ackerklee types in grass–clover mixtures. Grass and Forage Science 73, 297–308.
[8] Tork D.G., Anderson N. O., Wyse D. L. and Betts K. J. (2019): Domestication of perennial flax using an ideotype approach for oilseed, cut flower, and garden performance. Agronomy 9, 707.
[9] Zanotto S., Palmé A., Helgadóttir Á., Daugstad K., Isolahti M., Öhlund L., Marum P. Moen M. A., Veteläinen M., Rognli O. A. and Ergon Å. (2021): Trait characterization of genetic resources reveals useful variation for the improvement of cultivated Nordic red clover. Journal of Agronomy and Crop Science 207, 492–503.
[10] Nadeem S, Steinshamn H, Sikkeland EH, Gustavsson A-M, Bakken AK. 2019: Variation in rate of phenological development and morphology between red clover varieties: Implications for clover proportion and feed quality in mixed swards. Grass and Forage Science 2019; 74:403–414.

 DOI: 10.5507/vup.21.24459677.23

Enhancing Genetic Diversity through new Breeding Techniques

Susanne Barth[1] and Sergei Kushnir[1]

[1] Teagasc, Crops Research Centre, Carlow, R93XE12, Co. Carlow, Ireland

susanne.barth@teagasc.ie

Abstract. In this overview of New Breeding Technologies (NBT) we emphasize on the technological and new cultivar release challenges faced by the organizations that implement or plan to implement NBT. A focus is given on the unique types of genetic variation that can be created by NBT and never be found within natural or classically induced genetic variation available in crop and crop wild relative germplasms. We point out a little discussed NBT opportunity of a dramatic increase in the breeding targets within a specific program. We suggest that forage quality improvements are within reach.

Keywords: new breeding techniques, genome editing, genetic variation

1 Introduction

Genetic Diversity can be found in natural, semi-natural and breeding populations. In highly heterozygous genomes like in those of many forage species a very high degree of genetic diversity is observed and can be potentially exploited. The degree of exploitation by genetic introgression may be hampered in genomic regions, which are recalcitrant to genetic improvement. A typical problem is (i) suppressed meiotic recombination in peri-centromeric regions, or sites of large inversions; (ii) close physical map position to undesirable traits resulting in a linkage drag. Classic introgression of the natural genetic variation in breeding schemes, in itself, is also a time consuming step of crop improvement, which is particularly daunting process in species with a long seed-to-seed cycle, such as oil palm (5–6 years).

'New Breeding Techniques' (NBT) are under discussion and have made huge advances already in animal models and in plants. However, these new breeding techniques are since July 2018 considered under the ruling of the European Court of Justice as genetic modified organisms (GMOs) within the meaning of Directive 2001/18 and thus cannot find practical applications in farmers' fields unless they are deregulated as GMOs in the future.

New breeding techniques cover a wide range of technical systems [1] from Site-Directed Nucleases (SDN) (including zinc finger nuclease versions 1, 2 & 3; TALEN nucleases and CRISPR systems); Oligonucleotide Directed Mutagenesis (ODM); Cisgenesis; RNA-dependent DNA methylation (RdDM); Grafting (non-GM scion on GM rootstock); Reverse breeding and Agro-infiltration [2]. Currently in research the CRISPR system is the most favoured amongst the new breeding techniques, although genome editing of organelle genomes (chloroplast and mitochondrial DNA) can be achieved only by the designer DNA-modifying enzymes comprising TALEN DNA-binding domain.

For GM seeds, the legislation requires the varieties to be authorised in line with the procedures outlined in GMO Directive 2001/18/EC before they can be included in the Common Catalogue and be sold on the European market. If GM seed is intended to be used in food and feed, it has to follow the rules of Regulation (EC) 1829/2003 on genetically modified food and feed. The registration of GM seeds requires significant additional effort compared to non-GM seeds and will be only interesting for game changing applications.

2 Pre-requisites for the Application of New Breeding Techniques

The backbone of the application of new breeding techniques like CRISPR demands a high investment in science before it can be applied successfully. The application of genome editing can be started with the reasonable transcriptome data. However, a high quality genome sequence, including a decent coverage of difficult to assemble (peri)centromeric and telomeric regions, it is highly desirable. High quality genome assembly is especially needed if the approval process to release NBT-derived new cultivars into the field will demand an evidence of no off-target effects, and for exploration of unique breeding opportunities of the promoter and epigenome editing [3]. In many crop species no complete genomes are available, and if available sometimes not yet in the necessary assembly and annotation quality to allow the design of CRISPR components with minimal off-target effects. Work in progress with partially released or draft quality genomes in forages are e.g. white clover [4], red clover [5] and perennial ryegrass [6, 7]. However, even for these already well cared for forage species, the genomes are still not at a similar curation stage like for rice, wheat or barley, hence the design of CRISPR vectors is still hampered to some degree and errors can be made based on insufficient annotation status of genes. Forage species also drag far behind in quantification and annotation of copy-number (CNV) and presence-absence variation, the so-called pangenome. Likewise, the lack of pan-genome knowledge might hamper the NBT-generated cultivar approval.

How artificial DNA modifying enzymes are delivered into the plant cells to alter the forage species genome and epigenome is a technological challenge for the near future application. The most common approach especially suited for basic and proof of the concept types of research remains the expression from

transgenes encoding genome editing executing molecules, e.g. Cas9 protein and its guide RNA(s). Such so-called DNA delivery most commonly achieved either by *Agrobacterium*-mediated transformation and microprojectile particle bombardment, e.g. in ryegrass [8, 9], respectively. There are several key complications with the DNA delivery. Firstly, at least one and two seed-to-seed cycles are required to segregate the transgene out in self-compatible and incompatible, such as ryegrass, forages, respectively. Secondly, both DNA delivery methods often result in off-target genomic rearrangements; integrations of vector backbone, beside the T-DNA; multiple insertions lending support for the whole genome confirmation of no off-target effects, which further increases costs. Thus, the delivery of the proteins and/or RNA is more advantageous. Also genome ploidy matters. Segregating out the transgene is either impractical or impossible in (allo)polyploid or/and vegetatively propagated commercial cultivars, such as potato [10]. Thus, NBT in forages, e.g. Napier grass, sugar cane, white clover, better adapt RNA or protein delivery. RNA delivery is the most common in biomedical research, and largely relies on lipid nanoparticle (LNP) RNA packaging, currently also used to deliver COVID-19 RNA vaccines (Pfizer and Moderna). We are not aware of highly efficient LNP-mediated RNA delivery examples in plants, although plant viral delivery systems do work to some extent [11]. The key challenge with plant viral vectors is an induction of heritable changes when infecting a whole plant. Protein delivery is usually achieved either by particle bombardment [12] or by the polyethylene glycol (PEG)-mediated transformation [10]. Choosing the delivery method must consider the desired timing of the DNA breaks generation. It is thought that if two DNA breaks must happen within the same timeframe window during DNA repair, then Cas9/guide RNA ribonucleic acid protein (RNP) complex is the best means [12]. For example, induction of inversions, translocations, and large deletions does require two simultaneous DNA breaks. The delivery of RNP complex of CRISPR/Cas9 to maize enabled a 75.5 Mbp inversion necessary to restore meiotic recombination comprising traits of economic interest. This study is also an excellent example of the NBT power in producing breeding material that can be never achieved by the classic breeding techniques, i.e. precise restoration of the synteny in a genomic region of the interest.

Why would we plan and lobby for the societal acceptance of the NBT? Some types of genetic changes are simply a unique power of genome editing, like megabase-pair scale inversions [12], promoter [13] and epigenome editing [3]. NBT promises to skip genetic introgression of natural genetic variation in breeding schemes. Genetic introgression can take years of laborious crosses and phenotyping efforts. With genome editing, we can introduce desirable change in either any variety already on a market, or in the best breeding materials, within one seed-to-seed cycle. There is a hurdle to this promise, the daunting process of plant cell culture often required in NBT technologies. In collaborations with basic science, forage breeders should embrace, develop and explore all the tools to suppress plant cell culture recalcitrance [13]. Better still, breeders should at-

tempt new, daring, "out of the box" approaches to skip in vitro plant cell, tissue and organ culture altogether [14]. Only by doing that, we will gain tremendous savings in time and money during breeding strategies relying on NBT.

3 Traits for Improvement

Given enough time, funding and implementation of NBT, myriads of traits can be combined as targets in a breeding scheme, aiming at the golden standard of natural selection. In practice, however, breeders prefer to limit themselves to just a few traits, especially in frugally funded breeding programs. Thus, trait prioritization becomes of critical importance. Biomass yield and quality and biomass digestibility can be considered as top priorities that are within a few years reach if we adopt NBT.

The classic approach to enhance yield by hybrid breeding is hardly applied in forage crops. NBT can enable forage hybrid breeding. Genetic male sterility, breakdown of self-incompatibilities for implementation of the Seed Production Technology are easy to generate by NBT. Forage dihaploid generation can be achieved by genome editing for development of haploid inducers. Promoter editing in genes controlling shoot meristem size and shoot architecture [15, 16] could both increase the number of leaf primordia, and enlarge leaf size. Older leaf senescence, especially in the "three leaf" species, such as ryegrass, can be either eliminated or delayed, improving biomass quality. Classic GMO research showed practical ways to enhance of triacylglycerol; carbohydrate and protein contents in a leaf biomass. NBT could mimic those GMO engineering feats by modulating the species own genes in their genomic context. Promoter editing and genetic redundancy reduction by gene knock-out in lignin biosynthesis network can gradually decrease lignin content in forage biomass without compromising plant survival in the agroecosystems. Lignin-rich flowering stalks can be entirely eliminated by constructing never flowering forage cultivars.

References

[1] European Parliamentary Research Service: *New plant-breeding techniques: Applicability of GM rules,* 2016, https://www.europarl.europa.eu/RegData/etudes/BRIE/2019/642235/EPRS_BRI(2019)642235_EN.pdf, last accessed 2021/06/16

[2] Torti S., Schlesier R., Thümmler A., Bartels D., Römer P., Koch B., et. al., Gleba Y. (2021): Transient reprogramming of crop plants for agronomic performance. *Nature Plants* 7(2), 159–171.

[3] Nakamura M., Gao Y., Dominguez A.A., Qi L.S. (2021): CRISPR technologies for precise epigenome editing. *Nature Cell Biology* 23(1), 11–22.

[4] Griffiths A.G., Moraga R., Tausen M., Gupta V., Bilton T.P., Campbell M.A., et al., Uggerhøj Andersen S. (2019): Breaking Free: The genomics of allopolyploidy-facilitated niche expansion in white clover. *The Plant Cell* 31, 1466–1487.

[5] De Vega J.J., Ayling S., Hegarty M., Kudrna D., Goicoechea J.L., Ergon A., et al. Skøt L. (2015): Red clover (*Trifolium pratense* L.) draft genome provides a platform for trait improvement. *Scientific Reports* 5:17394.

[6] Byrne S.L., Nagy I., Pfeifer M., Armstead I., Swain S., Studer B., et. al., Asp T. (2015): A synteny-based draft genome sequence of the forage grass *Lolium perenne*. *Plant Journal* 84, 816–26.

[7] Velmurugan J., Mollison E., Barth S., Marshall D., Milne L., Creevey C.J., et al., Milbourne D. (2016): An ultra-high density genetic linkage map of perennial ryegrass (*Lolium perenne*) using genotyping by sequencing (GBS) based on a reference shotgun genome assembly. *Annals of Botany* 118, 71–87.

[8] Bajaj S., Ran Y., Phillips J., Kularajathevan G., Pal S., Cohen D., Elborough K., Puthigae S. (2006): A high throughput Agrobacterium tumefaciens-mediated transformation method for functional genomics of perennial ryegrass (*Lolium perenne* L.). *Plant Cell Reports* 25, 651–9.

[9] Zhang Y., Ran Y., Nagy I., Lenk I., Qiu J.L., Asp T., Jensen C.S., Gao C (2020): Targeted mutagenesis in ryegrass (*Lolium* spp.) using the CRISPR/Cas9 system. *Plant Biotechnology Journal* 18, 1854–1856.

[10] Andersson M., Turesson H., Olsson N., Fält A.S., Ohlsson P., Gonzalez M.N., Samuelsson M. , Hofvander P. (2018): Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. *Physiologia Plantarum* 164, 378–384.

[11] Ellison E.E., Nagalakshmi U., Gamo M.E., Huang P.J., Dinesh-Kumar S., Voytas D.F. (2020): Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs. *Nature Plants* 6, 620–624.

[12] Schwartz C., Lenderts B., Feigenbutz L., Barone P., Llaca V., Fengler K., Svitashev S. (2020: CRISPR–Cas9-mediated 75.5-Mb inversion in maize. *Nature Plants* 6, 1427–1431.

[13] Liu L., Gallagher J., Demesa Arevalo E.D., Chen R., Skopelitis T., Wu Q., Bartlett M., Jackson D. (2021): Enhancing grain-yield-related traits by CRISPR–Cas9 promoter editing of maize CLE genes. *Nature Plants* 7, 287–294.

[14] Maher M.F., Nasti R.A., Vollbrecht M., Starker C.G., Clark M.D., Voytas D.F. (2020): Plant gene editing through de novo induction of meristems. *Nature Biotechnology* 38, 84–89.

[15] Debernardi J.M., Tricoli D.M., Ercoli M.F., Hayta S., Ronald P., Palatnik J.F., Dubcovsky J. (2020): A GRF–GIF chimeric protein improves the regeneration efficiency of transgenic plants. *Nature Biotechnology* 38, 1274–1279.

[16] Wang H., Niu L., Fu C., Meng Y., Sang D., Yin P., et. al., Lin H. (2017): Overexpression of the WOX gene STENOFOLIA improves biomass yield and sugar release in transgenic grasses and display altered cytokinin homeostasis. *PLoS Genetics* 13:p.e1006649.

DOI: 10.5507/vup.21.24459677.24

Inheritance of self-fertility in tetraploid red clover

Tim Vleugels[1], Isabel Roldán-Ruiz[1,2] and Gerda Cnops[1]

[1] Plant Sciences Unit, ILVO (Flanders Research Institute for Agriculture, Fisheries and Food), Caritasstraat 39, BE-9090 Melle, Belgium
[2] Department of Plant Biotechnology and Bioinformatics, Ghent University, Technologiepark 71, BE-9052 Zwijnaarde, Belgium

tim.vleugels@ilvo.vlaanderen.be

Abstract. Tetraploid red clover (*Trifolium pratense* L.) is highly productive and persistent, but often displays unsatisfactory seed yield. Previous research in tetraploid red clover suggested a possible association between self-fertility and seed yield. In this paper, we investigated the inheritance of the self-fertility trait in tetraploid red clover. Self-compatible (SC) and self-incompatible (SI) genotypes were available from previous studies. Four inbred populations (S_1) were obtained through self-fertilization of SC genotypes. Eleven F_1 populations were obtained from crosses between SI and SC genotypes. Self-fertilization of SC genotypes yielded significantly more seeds than SI x SC crosses (5228 vs. 1125 seeds/plant respectively). Subsequently, S_1 and F_1 progeny populations were evaluated for their ability to self-fertilize. S_1 populations displayed 95% self-fertile plants, while F_1 populations displayed 69% self-fertile plants. In addition, S_1 populations yielded more seeds than F_1 populations when self-pollinated (401 vs. 149 seeds/plant respectively). These results clearly show that the ability to self-fertilize is a heritable trait in tetraploid red clover. To get more insights in the inheritance of self-compatibility, a subset of S_2 and F_2 populations has been selected for a next round of self-fertilization to be performed during summer 2021.

Keywords: *Trifolium pratense*, self-incompatibility, polyploidy, seed yield

1 Introduction

Red clover (*Trifolium pratense* L.) is a protein-rich forage crop that achieves high dry matter yield. It is an obligate out-crosser with a gametophytic self-incompatibility system controlled by a single S-locus [2]. Self-incompatibility is strongly expressed in diploid red clover, but acts weaker in tetraploid red clover [3], possibly due to the interaction of multiple alleles at the S-locus [1]. In previous research we demonstrated that self-fertilization does occur in open-pollinated tetraploid red clover genotypes that have been selected for high seed yield [5]. Congruently, in pair-crosses, high seed-yielding tetraploid genotypes displayed high degrees (up to 83%) of self-fertilization, whereas no self-fertilization was

observed in low seed-yielding tetraploids or in diploids [6]. Taken together, these studies indicate that a relatively high seed yield in some tetraploid genotypes might be the result of their ability to self-fertilize. Therefore, intensive selection for seed yield in tetraploid red clover may inadvertently lead to selection for increased self-fertility [5].

On the other hand, however, an increased degree of self-fertility in populations may lead to increased inbreeding. Yet little information exists on inbreeding effects in tetraploid red clover. Reductions in seed yield between 20 and 26% have been reported in genotypes resulting from self-pollination [4, 6]. No data is available for plant biomass or forage yield.

For breeders aiming to improve seed yield in tetraploid red clover, it is important to understand the associations between seed yield, self-fertility and inbreeding effects. In the present study, we investigated the inheritance of self-fertility in tetraploid red clover.

2 Materials and Methods

Four self-compatible (SC) and three self-incompatible (SI) tetraploid genotypes identified in previous research [6] were cloned and used to develop suitable populations. Inbred populations (S_1) were generated through self-pollination of the four SC genotypes. Additionally, 11 F_1 populations were obtained from pair-crosses between the three SI genotypes (as female parents) and the four SC genotypes (as male parents). As the three SI genotypes failed to form any seed when self-pollinated in previous studies, we assumed that all seed harvested on the SI genotype were the result of crossing with the SC genotype. Crosses were made in insect-proof gaze cages, spaced 1 x 1 m apart, in which 5 to 10 bumblebee drones (*B. terrestris*) were released weekly. Seeds were harvested individually on the SI plants.

In a subsequent trial, 15 progeny plants from each of the 4 S_1 and 11 F_1 populations (225 plants total) were planted in the field to check their ability to self-fertilize and to produce viable seeds which are the S_2 and F_2 populations. The 15 populations were established as rows of 15 individual plants, with planting distance 1 m within and between rows. Upon flowering, each plant was enclosed in an insect-proof nylon bag. To reduce labor, no insects were added during flowering, but plants were pollinated by daily shaking the nylon bags around noon. Seeds were harvested on a per-plant basis. Plants yielding more than 10 seeds were deemed self-compatible. Data were analyzed using ANOVA.

3 Results and Discussion

Self-fertilization of SC genotypes yielded more seeds than crosses between SI and SC genotypes (5228 vs. 1125 seeds/plant, respectively; $p < 0.001$) (Table 1),

which indicates that the SC plants are better seed producers than the SI plants. In one cross (Astur 15 x Taifun 15), no seeds were obtained because the SI plant perished before setting seed.

In the progeny trial, seed yields were substantially lower than in the original crosses, presumably because the parent trial was insect-pollinated while the progeny trial was not. While 95% of plants from the S_1 populations produced more than 10 seeds and were regarded as fully self-compatible, F_1 populations were only partly self-compatible with 69% self-compatible plants. The fact that these F_1 plants were able to produce seeds suggests that their SC male parents have transmitted the self-fertility trait to them. In other words, our results suggest that the ability to self-fertilize is a heritable trait in tetraploid red clover as postulated by Vleugels et al. (2019b) [6].

On average, self-pollinated S_1 plants produced 401 seeds, while self-pollinated F_1 plants produced only 149 seeds ($p < 0.001$). All S_1 plants produced more seeds than F_1 plants with the same SC ancestor (Table 1). This finding increases evidence for our previously suggested theory that self-fertility is associated with high seed production in tetraploid red clover, at least in poorly pollinated conditions [5].

To fully reveal the pattern of inheritance of self-fertility, a subset of S_2 and F_2 populations was selected for a next round of self-pollination, to be performed

Table 1. Seed yield after self-pollination of SC plants and crosses between SI and SC plants, seed yield of self-pollinated S_1 and F_1 progeny plants from these crosses, and the proportion of S_1 and F_1 plants yielding more than 10 seeds and regarded self-compatible.

Original crosses			Progeny trial		
Genotypes crossed	Type of cross	Average seed number per plant	Progeny pop.	Average seed number per plant	Proportion of plants yielding >10 seeds
Atlantis 07 x Atlantis 07	SC selfed	4988	S_1	292	100%
Astur 15 x Atlantis 07	SI x SC	777	F_1	279	73%
Avanti 13 x Atlantis 07	SI x SC	1510	F_1	167	87%
Avanti 18 x Atlantis 07	SI x SC	3010	F_1	112	73%
Taifun 15 x Taifun 15	SC selfed	6477	S_1	257	86%
Astur 15 x Taifun 15	SI x SC	0			
Avanti 13 x Taifun 15	SI x SC	355	F_1	208	80%
Avanti 18 x Taifun 15	SI x SC	273	F_1	66	71%
Tempus 07 x Tempus 07	SC selfed	2697	S_1	490	100%
Astur 15 x Tempus 07	SI x SC	1063	F_1	114	71%
Avanti 13 x Tempus 07	SI x SC	636	F_1	21	40%
Avanti 18 x Tempus 07	SI x SC	887	F_1	122	60%
Titus 15 x Titus 15	SC selfed	6751	S_1	565	93%
Astur 15 x Titus 15	SI x SC	2205	F_1	301	87%
Avanti 13 x Titus 15	SI x SC	1270	F_1	178	67%
Avanti 18 x Titus 15	SI x SC	1510	F_1	64	53%

during summer 2021. Finally, we plan to establish a spaced plant trial to quantify the effect of inbreeding by comparing plant biomass and seed yield over three generations of inbreeding (S_1, S_2 and S_3) and the four ancestral SC genotypes that are currently maintained as clones.

4 Conclusion

In our study, progeny obtained through self-pollination of 4 SC genotypes (S_1 populations) displayed 95% self-fertile plants, while progeny obtained through crossing the same 4 SC genotypes with 3 SI genotypes (F_1 populations) displayed only 69% self-fertile plants. These findings suggest that self-fertility is a heritable trait in tetraploid red clover. In future research we will investigate our hypothesis that self-fertility is governed by a single gene.

Our study also revealed that S_1 populations yielded substantially more seeds than F_1 populations with the same SC ancestor, which increases evidence that self-fertility may be associated with high seed yield in tetraploid red clover. The effects of inbreeding on plant biomass and seed yield remain to be studied in a future spaced-plant trial.

References

[1] Barringer B.C. (2007): Polyploidy and self-fertilization in flowering plants. American *Journal of Botany* 94, 1527–1533.

[2] Boller B., Schubiger F.X. and Kölliker R. (2010): Red clover. In: Boller B., Posselt U.K., Veronesi F. (eds) Fodder Crops and Amenity Grasses, Springer New York, New York, pp. 439–455.

[3] Grebrenisan M. and Savatti, M. (2011): The comparative effects of the micro- and macrosporogenesis of the red clover with different levels of ploidy, in relation with its fertility. *Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Animal Science and Biotechnologies* 68, 138–143.

[4] Laczynska-Hulewicz T. (1963): Self-fertility and inbreeding in tetraploid red clover. *Genetica Polonica* 4, 97–119.

[5] Vleugels T., Roldán-Ruiz I. and Cnops G. (2019a): Marker-assisted parentage analysis reveals high individual selfing rates in tetraploid red clover genotypes selected for seed yield. *Plant Breeding* 138, 947–957.

[6] Vleugels T., van Laere K., Roldán-Ruiz I. and Cnops G. (2019b): Seed yield in red clover is associated with meiotic abnormalities and in tetraploid genotypes also with self-compatibility. *Euphytica* 215, 79.

DOI: 10.5507/vup.21.24459677.25

Genomic prediction of lucerne forage yield and quality

Marie Pégard[1], Julien Leuenberger[1], Bernadette Julier[1] and Philippe Barre[1]

[1] INRAE P3F, 86600 Lusignan, France

marie.pegard@inrae.fr

Abstract. Genomic prediction has proven its efficiency in numerous animal and plant species. In this study, we used diverse lucerne varieties and populations to test the predicting ability of genomic prediction. Several parameters, such as the number of markers, the population size and the addition of QTL effects, were tested for their effect on the quality of prediction. Based on a large number of SNPs (227 K) obtained by GBS and phenotypes observed in different locations, our results showed a good quality of predicting ability for dry matter yield, ADF (acid detergent fiber) and protein content, especially with a large training population size (around 0.6). The predicting ability is improved by the integration of QTL information directly in the model (above 0.8). A reduction of number of markers (less than 100K) did not substantially alter the predictive ability. Our results show an accurate prediction of the phenotype of populations *via* genomic prediction models that could speed up the creation of new lucerne varieties.

Keywords: alfalfa, genomic selection, GBS.

1 Introduction

Lucerne (*Medicago sativa* L.) is an important crop worldwide, it plays a major role in farm protein autonomy and also offers multiple ecosystem services. It is a cross-pollinating, auto-tetraploid species, which makes its improvement more complex. Various authors have shown the interest of genomic selection in plant and animal species. Genomic selection can be defined as a prediction of the breeding values based on the genotypic data to select the best individuals for further breeding. Some studies have been published on genomic evaluation of lucerne. They report poor or moderate accuracies (0–0.65) for yield and quality traits, with a number of SNPs from 8K to 44K and a number of individuals between 75 and 274 [1] [4]. In the present study, we evaluated the ability of genomic prediction models coupled with QTL detected by GWAS (Genome Wide Association Study) to predict the phenotype of diverse genetic resources

composed by 400 populations (pools of individuals), genotyped with a large number of markers (227K SNPs).

2 Material and Methods

The 400 lucerne accessions comprised cultivars, advanced breeding material and landraces. The accessions were genotyped by genotyping-by-sequencing (GBS) in pools of a hundred individuals and the reads were mapped on the reference genome [5]. After the application of several quality filters, 227K SNP were obtained. The 0.57 % of missing values were imputed by the minor allele frequency. Phenotypic data of the 400 accessions (dry matter yield, acid detergent fiber (ADF) and protein content) were obtained in trials established in 2018 at three locations (Lusignan - FRA, Novi Sad - SRB, Store Heddinge – DNK) and scored during two years (2019 and 2020). The phenotypes were adjusted for the micro-environmental variation inside each trial, the year and the location effect. Cross-validation was used to assess the prediction quality combined with an independent Test Set representing 20% of the 400 populations (80 populations among the 400). We tested the effect of different population sizes (75%, 50% or 25% of the 320 remaining accessions), and the number of markers on the prediction ability. Several sets of markers corresponding to different marker densities were tested. Based on a window of a given physical size, five sets of markers were designed: 74K, 29K, 12K, 5K and 1.3K SNP. We also tested the influence of integrating QTL information in the model as fixed effects (QGBLUP) compared to the classical GBLUP model. The QTL were detected by Multi-Locus Mixed Model approaches [6]. The predicting ability computed as the Pearson's correlation between the observed phenotype and the predicted value, provided an estimate of the prediction quality.

3 Results

The prediction quality was relatively high: between 0.30 and 0.80 (Fig. 1A). A decrease in the size of the training population had a modest negative impact on the quality of the prediction, whatever the trait.

The GWAS analysis revealed 19 QTL for dry matter yield (explaining between 0.016% and 13.5% of the phenotypic variation), 15 for ADF content (explaining between 0.09 % and 8.6 %) and 15 for protein content (explaining between 0.003% and 15.9 %). The addition of these QTL to the prediction model improved the prediction quality above 0.80 when large training populations were used (Fig. 1A).

The results obtained with the different marker sets showed little impact of the number of markers on the predicting ability (Fig. 1B). The data set with the lowest numbers of markers (1397), however, clearly showed a lower predic-

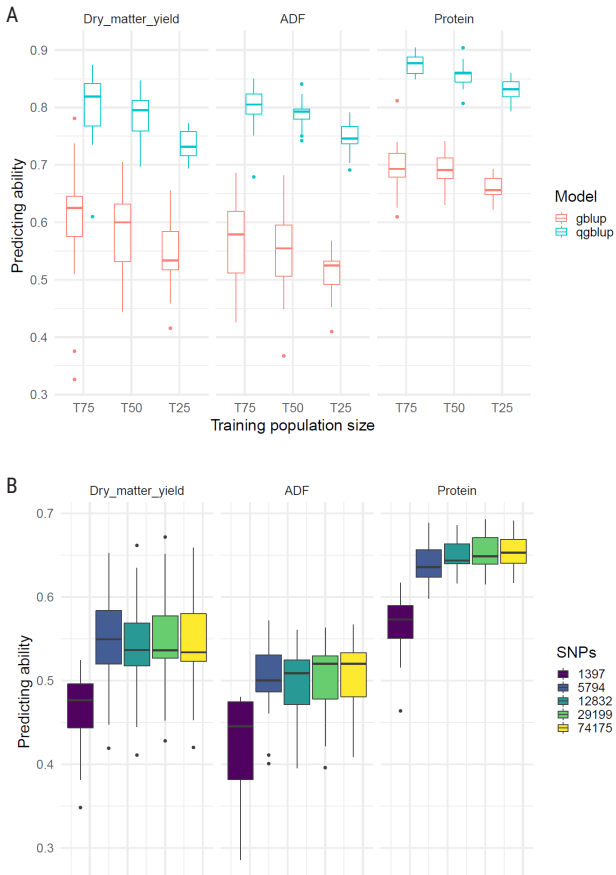


Fig. 1. Predicting of the Test Set (80 accessions) for three traits (dry matter yield, ADF and protein content). **A** : Predicting ability depending on the size of the training population. Two models are presented; the GBLUP in pink and the QGBLUP in blue where QTL are integrated in the model as fixed effects. **B** : Predicting ability of the T50 training population size depending on the number of markers used in the model.

tive ability than the sets with a higher number of markers. There was a plateau between the set of 5,794 markers (1 marker every 100,000 bases) and the set of 74,175 markers (1 marker every 50 bases). A limited number of markers seems to be sufficient to reach a good quality of prediction in this present dataset and in these conditions (less than 100K). However, a large number of markers was required to detect the QTL that increased the quality of prediction. We can imagine to use a large number of markers to detect the QTL and then combine them with a small set of markers for the routine prediction. This would accelerate

the improvement of lucerne and facilitate the creation of new cultivars. The next step will be to see if these prediction equations accurately predict the current elite material of breeders.

References

[1] P. Annicchiarico, N. Nazzicari, X. Li, Y. Wei, L. Pecetti, and E. C. Brummer, 'Accuracy of genomic selection for alfalfa biomass yield in different reference populations', *BMC Genomics*, vol. 16, no. 1, p. 1020, Dec. 2015, doi: 10/f8ddkp.

[2] E. Biazzi *et al.*, 'Genome-Wide Association Mapping and Genomic Selection for Alfalfa (Medicago sativa) Forage Quality Traits', *PLoS ONE*, vol. 12, no. 1, p. e0169234, Jan. 2017, doi: 10/f9kvzb.

[3] C. Jia *et al.*, 'Genomic Prediction for 25 Agronomic and Quality Traits in Alfalfa (Medicago sativa)', *Front. Plant Sci.*, vol. 9, 2018, doi: 10/gjjz78.

[4] X. Li *et al.*, 'Genomic Prediction of Biomass Yield in Two Selection Cycles of a Tetraploid Alfalfa Breeding Population', *Plant Genome*, vol. 8, no. 2, Jul. 2015, doi: 10/ggjp3c.

[5] H. Chen *et al.*, 'Allele-aware chromosome-level genome assembly and efficient transgene-free genome editing for the autotetraploid cultivated alfalfa', *Nature communications*, vol. 11, no. 1, pp. 1–11, 2020.

[6] V. Segura *et al.*, 'An efficient multi-locus mixed-model approach for genome-wide association studies in structured populations', *Nature genetics*, vol. 44, no. 7, p. 825, 2012, doi: 10/f34dkw.

DOI: 10.5507/vup.21.24459677.26

Impact of genetic relatedness on the genomic prediction accuracies in timothy (*Phleum pratense* L.)

Mallikarjuna Rao Kovi[1], Akhil Reddy Pashapu[1], Helga Amdahl[2], Muath Alsheikh[2], Peter Marum[2] and Odd Arne Rognli[1]

[1] Department of Plant Sciences, Faculty of Biosciences, Norwegian University of Life Sciences, Ås, Norway
[2] Graminor AS, Ridabu, Norway
[3] Department of Molecular Biology and Genetics, Aarhus University, Slagelse, Denmark

mallikarjuna.rao.kovi@nmbu.no

Abstract. Timothy (*Phleum pratense* L.) (2n=6x=42), a cool-season perennial grass species, is one of the most important forage grass species in Norway because of its good winter hardiness, ability to thrive in short-growing season and resistance to frost and ice-encasement. However, the predicted increase of temperatures during autumn and winter at high latitudes in the coming decades and the associated variable temperature and precipitation patterns might negatively affect cold acclimation and winter survival of perennial grasses, therefore potentially affecting the forage production in Nordic regions. New improved cultivars with high yield capacity and forage quality, which are well adapted to the future climate, are very important for an economically sustainable Norwegian milk and meat production. In the view of the recent developments in sequencing, molecular marker technologies and theoretical foundations, it is feasible to start the development of genomic selection (GS) based breeding schemes in timothy. In this paper, we tested a clustering-based approach to investigate the effect of increasing genetic distance between training and validation populations when predicting yield traits. The prediction accuracies in the validation population highly depend on the genetically closely related training population, irrespective of the population size. This approach is valuable in addressing an important point on how to structure the training population to maximise the prediction accuracy in genomic selection models.

Keywords: genomic selection, forage grass, prediction accuracy

1 Introduction

Forages are the economically most important crops in Norway, and timothy seeds constitute more than 50% of the seed sold for establishing leys for forage production. A combined effort of improved agronomy and breeding of high-

yielding and persistent cultivars is needed in order to secure stable supplies of high-quality forages for meat and milk production. Forage yield (which highly depends on climate adaptation, winter survival and persistency) and herbage quality, are the two main traits determining the economic value of herbage from perennial grasses in livestock production. Improving both these traits lead to superior cultivars, contributing to a more efficient, sustainable, and economically viable livestock farming. However, improving these key traits is laborious and requires multi-environment trials over several years. Therefore, there is a great need to develop and implement new breeding technologies in timothy breeding. Genomic selection (GS) is a method that combines molecular markers with phenotypic and pedigree data to predict breeding values based on all available markers throughout the genome [1]. With recent developments in genomic information, it is feasible to start the development of GS-based breeding schemes also for timothy. However the key question is how to structure the training population to maximise the prediction accuracy in genomic selection models. The objectives of this study were 1) to investigate the correlations between genomic estimated breeding values (GEBVs) and actual observed phenotype values for yield traits in timothy; and 2) to explore the impact of genetic relatedness on the genomic prediction accuracies.

2 Materials and Methods

A total of 847 second generation full-sib families (FS-2) were available at the breeding station of Graminor AS. These FS-2 families were progeny tested (2003–2013) in field trials at two locations in Southern Norway, at Bjørke, Hamar – a lowland location, and at Løken, Valdres – a highland location. Yield data were available for 3 harvest years with 2 or 3 cuts per year. Herbage quality data, i.e. crude protein, in vitro digestibility, NDF, ADL, water-soluble carbohydrates, and various estimates of energy concentration, were available for each of 3 cuts of 630 families, sampled at Bjørke in a single year. A total of 216 newly developed FS-2 families were sown at Bjørke and Løken, and complete date for forage yield and herbage quality were available. These 216 families were genotyped, and 200 families were used as validation population to cross-validate GS models to estimate the prediction accuracy. All FS-2 families were sequenced using a robust next generation sequencing technology "Genotyping-by-sequencing" (GBS) [2]. The reads were aligned to the draft genome of timothy (unpublished) and the SNP calling was performed using STACKS reference-based pipeline [3]. Further, we used SNP filtering methods such as missing proportion of markers at an individual ≥ 40% and missing proportion of markers at loci ≥ 15%. RR–BLUP, Bayes C and Bayesian LASSO (BLASSO) approaches were implemented to estimate the GEBV for dry matter yield and forage quality data. In practice, GBS should allow to predict traits in families with related genetic material that has been genotyped and phenotyped. We tested the hypothesis that retaining only

the most closely related genotypes or clusters in the training set might maximize prediction accuracies and obtained accuracy values for validation set and trait using two different training set sizes and compositions.

We simulated training and validation samples with varying genetic distances by splitting the training population into a sequence of pairs of subsets with increasing genetic differentiation. Different genomic selection models for yield and quality traits were evaluated.

3 Results and Discussion

In total, we obtained 60,635 SNPs with minimum minor allele frequency of 0.05. The resulting final marker dataset of 30,698 SNPs was used for genomic predictions. Our results show that genomic prediction has worked well in timothy for yield-related traits. The predictive ability plot (Figure 1; Table 1) for dry matter yield (DMY) for three cuts in three years shows good correlation between the GEBV and the observed phenotypes in the validation set. The accuracies (correlation between the genomic estimated breeding values and the observed

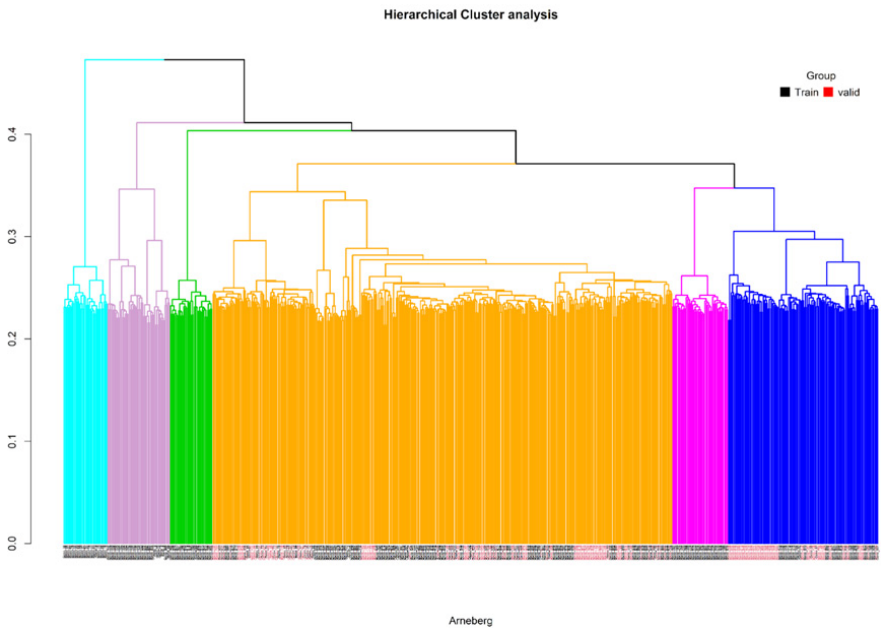


Fig 1. Genetic relatedness among the training and testing families (full-sib families) in Timothy. 613 full-sib families (413 training and 200 validation set) from one location (Arneberg, Norway) clustered in 6 groups based on their genetic distance. The black labels on X-axis represent the training set, the red labels represent the validation set.

Table 1. Prediction accuracies for dry mater yield with two different training sets: 413 and 259 full sib families respectively (only selected the full sib families from the closely related genetic clusters) and a validation set of 200 families. DMY101, DMY102: dry matter yield for 1st cut, 2nd cut in the 1st year; DMY201, DMY202: dry matter yield for 1st cut, 2nd cut in the 2nd year; MDY301: dry matter yield for 1st cut in the 3rd year; SUMDMY: total DMY over all cuts.

Training set	Validation set	DMY 101	DMY102	DMY201	DMY202	DMY301	SUMDMY
413	200	0.18	0.08	0.50	0.20	0.01	0.32
259	200	0.18	0.09	0.54	0.25	-0.03	0.34

phenotypes) in the validation population were similar when excluding families that were not genetically closely related from the training population (Table 1).

4 Conclusion

Our study showed good prediction accuracies in timothy for a couple of forage yield recordings (0.54 for DMY first cut second year; 0.25 for total DMY second year). However, predictions of other yield recordings and traits resulted in very low correlations, in the same range as detected by Grinberg *et al.* (2016) [4] in perennial ryegrass. Further, our genomic prediction results showed that the prediction accuracies in the validation population highly depended on the genetically closely related training population, irrespective of the population size.

Acknowledgement

The research leading to these results received funding from the Norwegian research council (NFR)

References

[1] Meuwissen T.H., Hayes B.J. and Goddard M.E. (2001): Prediction of total genetic value using genome-wide dense marker maps. *Genetics* 157, 1819–1829.

[2] Elshire R.J., Glaubitz J.C., Sun Q., Poland J.A., Kawamoto K., Buckler E.S., and Mitchell S.E. (2011): A Robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PLoS ONE* 6, e19379.

[3] Catchen J., Hohenlohe P., Bassham S., Amores A. and Cresko W. (2013): Stacks: an analysis tool set for population genomics. *Molecular Ecology* 22(11), 3124–3140.

[4] Grinberg N. F., Lovatt A., Hegarty M., Lovatt A., Skøt K. P., Kelly R., Blackmore T., Thorogood D., King R. D., Armstead I., Powell W. and Skøt, L. (2016): Implementation of genomic prediction in *Lolium perenne* breeding populations. *Frontiers in Plant Science* 7, 133.

DOI: 10.5507/vup.21.24459677.27

Feature selection for genomic prediction of perennial ryegrass forage quality

Agnieszka Konkolewska[1], Patrick Conaghan[2], Dan Milbourne[1], Michael Dineen[2], Susanne Barth[1], Rachel Keirse[1] and Stephen Byrne[1]

[1] Teagasc, Crop Science Department, Oak Park, Carlow, R93 XE12, Ireland
[2] Teagasc, Grassland Science Research Department, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Cork, Ireland

agnieszka.konkolewska@teagasc.ie

Abstract. Feature selection enables the identification of important SNPs for development of low-density genotyping assays for use in genomic selection. The objective of this study was to evaluate genetic algorithms for feature selection prior to genomic prediction of forage digestibility in *Lolium perenne*. The reference population consisted of 1800 genotypes from 30 diploid populations consisting of 10 cultivars, 8 full-sib families, 8 half-sib families and 4 ecotypes. Plants were established in a spaced plant field trial and forage dry matter digestibility (DMD) was determined with near-infrared spectroscopy (NIRS). Genomic predictions based on genotyping-by-sequencing (GBS) data were performed on a full marker dataset and reduced marker subsets. Genetic algorithms enabled us to select subsets of features with predictive ability comparable to the entire SNPs set. While further optimization is required, identifying key predictor variables may enable selection for forage quality using inexpensive marker systems.

Keywords: Genomic Selection, Plant Breeding, Feature selection

1 Background

Genomic selection (GS) has the potential to accelerate genetic gain for complex forage traits in perennial ryegrass breeding programs [5]. However, genotyping at high densities to calculate Genomic Estimated Breeding Values (GEBVs) on selection candidates can be cost prohibitive to apply GS routinely. Feature selection for development of low-density genotyping assays to calculate GEBVs might be a solution.

Genetic algorithm (GA) is a heuristic approach that mimics natural selection processes in nature, such as mutation, crossover and selection, for feature selection [6]. In this study, we evaluate the implementation of GA to select re-

duced number of SNP markers for prediction of forage DMD, without the loss of predictive ability.

2 Methods

The training population (1800 genotypes) consisted of 30 diploid perennial ryegrass populations comprising 60 genotypes each [1]. Plants were grown in two replicates. Quality data comes from the 1st cut of the 2015. NIRS calibrations were used to predict feed quality traits, including DMD [2]. Predicted quality values were averaged per genotype. Plants were genotyped-by-sequencing (GBS) as shown by Elshire et al. (2011) [3] and data analyzed as in Arojju et al. (2018) [1]. Genotypes with low sequencing coverage were eliminated. Markers with more than 25% missing data were removed and the missing data for markers that were retained in the analysis was mean imputed. Ridge regression best linear unbiased prediction (rrBLUP) was used for genomic prediction in R with the rrBLUP package [4] on a full set of markers (~18,000). Genomic prediction models were evaluated using Monte-Carlo cross-validation by randomly assigning plants into training (70%) and test (30%) sets (1,000 iterations). Predictive ability (PA) was determined as the Pearson's correlation coefficient between observed phenotypic value and predicted phenotype. Bias was evaluated by regressing observed phenotypic value on predictions.

A genetic algorithm was implemented in R to select subsets of features (SNPs) most relevant for genomic prediction. The implementation proceeded as follows: let U be a set that contains full SNPs dataset. For $n = 200$ SNPs (1% of a full set of markers, rounded up), $k = 50$ subsets of size n are randomly selected from U. For each k subset, genomic prediction with rrBLUP is performed using 10-fold cross-validation. Obtained predictive ability (PA), bias and interquartile range (IQR) are averaged over all folds. Next, subsets are ordered in descending order based on a score calculated as 0.5PA+0.5(1-IQR). Each subset (A) is paired with a randomly selected different (B) subset. Two random split sites are generated per subset and SNPs falling between split sites are exchanged between A and B, forming recombined A' and B'. Each marker in the recombined subsets can be substituted with a randomly selected marker (e.g. for A' new SNPs can be drawn from $U - A$'), with mutation probability $p = 0.01$. New subsets are used as input for rrBLUP, with 10-fold cross-validation. The resulting marker subsets are ordered in descending order for calculated score, and first k subsets are kept for the next iteration. We used leave-one-population-out fold-validation to assess the performance of selected SNP subsets on unseen data: for each fold, (i.) the genetic algorithm was run on a training set excluding one population, (ii.) a prediction model was built on the training data, and (iii.) GA selected markers and full SNPs dataset were used to predict DMD values on unseen data. The PA and bias were calculated.

3 Results

The mean predictive ability and bias of 1,000 iterations of Monte-Carlo cross-validation for DMD for the full marker set was 0.42 and 1.0, respectively. In different runs of genetic algorithm, the best 200 SNP set from the initial 50 subsets would have a mean PA of around 0.32. After 10 iterations, the best subset (GA) would usually reach PA level of 0.4, with more iterations leading to overfitting. Under a more challenging leave-one-population-out validation, the PA varied greatly depending on the population being left out (Fig. 1), emphasizing the importance of a relationship between training and testing set. In the case of the genetic algorithm, the GA subset obtained lower mean PA, when used to predict DMD on unseen data, compared to a full SNPs dataset. In general, higher PA was obtained for populations that were genetically closer to populations from the training set.

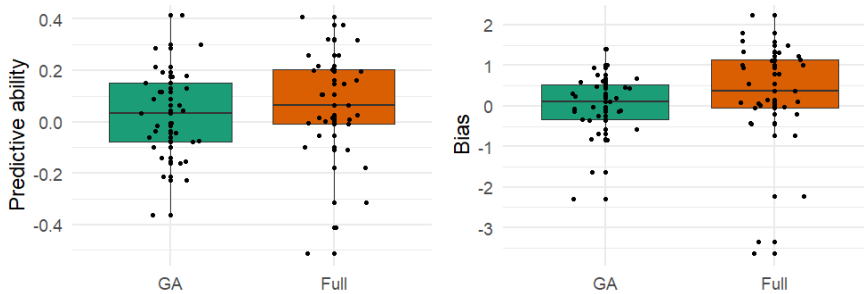


Fig 1. Predictive ability and bias of marker subset selected by genetic algorithm (GA, 200 SNPs, 10 iterations) and a full dataset (17974 SNPs) under leave-one-population-out validation.

Further work will be done to optimize selection of the size of SNPs subsets and the genetic algorithm itself, to limit the risk of overfitting. Other plans include a comparison of the GA results with other feature selection methods, such as genome-wide association studies (GWAS), as well as a validation of predictive ability of selected SNPs subsets on the F2 populations. Identifying a small subset of features with high predictive ability will enable selection for forage quality using inexpensive marker systems.

Acknowledgement

AK is funded from the European Union's Horizon 2020 program (Marie Sklodowska-Curie grant agreement No. 841882). This work is also supported by a research grant from Science Foundation Ireland and the Department of Agriculture, Food and the Marine on behalf of the government of Ireland under the grant 16/RC/3835 (VistaMilk).

References

[1] Arojju S.K., Conaghan P., Barth S., Milbourne D., Casler M.D., Hodkinson T.R., ... Byrne S.L. (2018): Genomic prediction of crown rust resistance in *Lolium perenne*. *BMC Genetics* 19(1), 35.
[2] Burns G.A., Gilliland T.J., McGilloway D.A., O'Donovan M., Lewis E., Blount N., O'Kiely P. (2010): Using NIRS to predict composition characteristics of *Lolium perenne* L. cultivars. *Advances in Animal Biosciences* 1(1), 321–321.
[3] Elshire R.J., Glaubitz J.C., Sun Q., Poland J.A., Kawamoto K., Buckler E.S., Mitchell S.E. (2011): A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PLoS ONE* 6(5), e19379.
[4] Endelman J.B. (2011): Ridge regression and other kernels for genomic selection with R package rrBLUP. *The Plant Genome* 4(3), 250–5.
[5] Hickey J.M., Chiurugwi T., Mackay I., Powell W., Eggen A., Kilian A., ... Beyene Y. (2017): Genomic prediction unifies animal and plant breeding programs to form platforms for biological discovery. *Nature Genetics* 49(9), 1297–1303.
[6] Whitley D. (1994): A genetic algorithm tutorial. *Statistics and computing* 4, 65–85.

DOI: 10.5507/vup.21.24459677.28

Italian Ryegrass – Higher Seed Yield through Targeted Selection for Low Seed Shattering

Jenny Peter[1,2], Roland Kölliker[2], Bruno Studer[2] and Cristoph Grieder[1]

[1] Agroscope, Fodder Plant Breeding, Reckenholzstrasse 191, CH-8046 Zurich
[2] Molecular Plant Breeding, Institute of Agricultural Sciences, ETH Zurich, Universitaetstrasse 2, CH-8092 Zurich

jenny.peter@agroscope.admin.ch

Abstract. For a forage grass cultivar to be successful on the market, a high seed yield is a prerequisite. Therefore, the main goal of this project is to evaluate the feasibility of including seed yield as a selection criterion in Italian ryegrass breeding. A preliminary test with Agroscope germplasm showed that reduced seed yield was mainly caused by seed shattering, which is thought to be controlled by only a few genes. Currently, a field experiment is performed to examine the potential of reducing seed shattering by direct phenotypic selection on spaced plants. Starting from a common base population, plants are selected for high and low seed shattering. After one cycle of selection, a significant difference was observed between families selected for high or low seed shattering, respectively. Therefore, selection for reduced seed shattering seems to be very effective in Italian ryegrass. A second selection cycle be performed and families will be evaluated as spaced plants and in rows.

Keywords: Seed yield, Seed shattering, Italian ryegrass, Phenotypic selection

1 Introduction

Italian ryegrass (*Lolium multiflorum* Lam.) is one of the most abundant forage grass species used for forage-based meat and dairy production in temperate areas. Besides delivering a good forage quality, a successful cultivar should have a high seed yield [6]. The potential seed yield (PSY) of a crop is defined as the total number of ovules per area present at flowering time [3]. Up to 40% of the PSY in forage grasses can be lost by suboptimal pollination, fertilization and seed development and by excessive seed shattering [3], resulting in a substantially lower realized seed yield (RSY). A preliminary experiment revealed that the difference between PSY and RSY was particularly high in the Italian ryegrass breeding material, mainly because of excessive seed shattering.

The heritability of seed yield per plant in perennial ryegrass (*Lolium perenne* L.) was shown to be high and direct phenotypic selection should be worthwhile in Italian ryegrass [1]. In addition, several easy to determine traits such as heading date were reported in several species to be correlated with high RSY, which would make them ideal targets for indirect selection [4] [5].

The objective of this study is (i) to determine whether direct selection for low seed shattering in Italian ryegrass can be successful to increase RSY and (ii) to identify target traits for indirect selection for low seed shattering in Italian ryegrass.

2 Materials and Methods

An experimental population consisting of 10 accessions of 30 plants each, was established in 2016. Single plants were phenotyped for seed shattering in 2017. For this, plants were bagged after flowering and harvested after a defined temperature sum by smoothly shaking their seeds in a bag and putting the plant with the remaining seeds still sticking to the tillers in a separate bag. Seed shattering was calculated as the weight of seeds lost (from the first bag) divided by total seed weight (seeds lost + seeds still on tillers). A first selection step was performed by selecting the 21 plants with highest and the 22 plants with lowest seed shattering. After allowing both groups of plants to openly pollinate in isolation, 20 genotypes per half-sib family (21 high seed shattering, 22 low seed shattering) were planted in the field in duplicates in a split-plot design with family as main-plot factor and genotype as sub-plot factor. Single plants were phenotyped for seed shattering, heading date, start flowering, plant vigour and stem rust (*Puccinia graminis* ssp. *graminicola*) infection for the second growth. Data were analysed using mixed models following the equation

$$Y = s_i + g_{ij} + r_k + m_n + \varepsilon_{ijkn},$$

where s_i is the selection applied (high or low seed shattering), g_{ij} is the effect of genotype j, r_k the replicate, m_n the effect of main plot n, and ε_{ijkn} the residual variation. Selection, genotype and replicate were treated as fixed and main plot as random factor. Broad-sense heritability was calculated as:

$$H = \sigma_g^2 / (\sigma_g^2 + \sigma_\varepsilon^2 / r),$$

where σ_g^2 is the variance of genotypes, σ_ε^2 is the error variance and r is the number of replicates. Correlations among seed shattering and the other observed phenotypic data were calculated as Pearson's correlation coefficient based on means per genotype.

3 Results and Discussion

After the first cycle of selection, a significant difference in seed shattering was observed between families selected for high or low seed shattering ($p = 0.015$; Fig. 1). Furthermore, the genotype had a highly significant effect ($p < 0.001$) on seed shattering. The broad-sense heritability of seed shattering in our population was 0.62. Thus, direct selection for low seed shattering in spaced plants in Italian ryegrass seems to be possible and effective. However, since quantifying seed shattering is very laborious and time consuming, indirect selection based on correlated traits could offer a promising alternative. Heading date, which is highly heritable in perennial ryegrass, is one of the important traits influencing RSY in forage grasses [2] [4]. In the present study, heading date and start of flowering were correlated ($r = 0.64$), as expected, but they were neither correlated with the amount of seeds still on the tillers nor with seed shattering (data not shown). However, more vigorous plants showed a higher total seed weight ($r = 0.53$), possibly due to more tillers and therefore a higher RSY, as demonstrated in perennial ryegrass [1]. Moreover, there was a negative correlation found between the seeds still on the tillers and seed shattering ($r = -0.75$). Although, further investigations are needed, seeds still on the tiller could be an easy and feasible trait to select indirectly for low seed shattering.

To conclude, selection for low seed shattering among spaced plants of Italian ryegrass is possible and worthwhile. Using the results of seed shattering from the 2020 experiment, we will perform a second cycle of selection. The repeated positive and negative selection will then be compared to a single positive and negative selection and an unselected offspring in single plants and row trials in 2022.

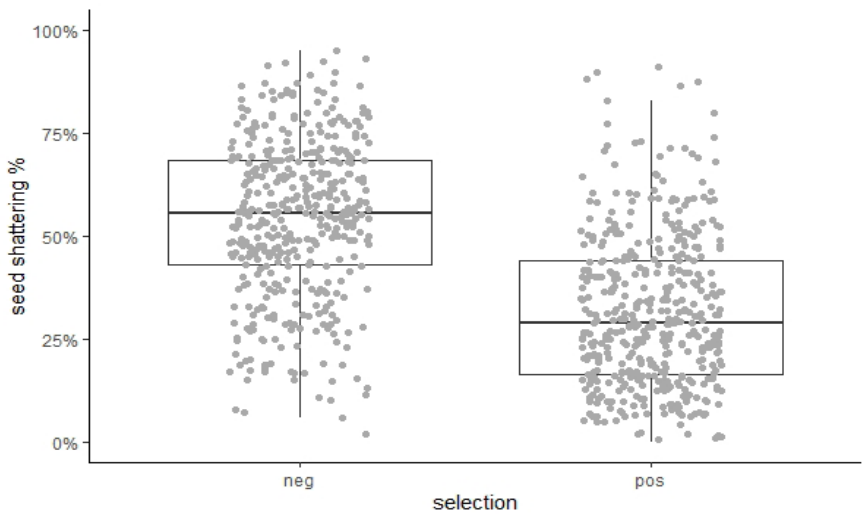


Fig. 1. Seed shattering for 21 Italian ryegrass families selected for high (neg) and 22 Italian ryegrass families selected for low (pos) seed shattering.

References

[1] Bugge G. (1987): Selection for seed yield in Lolium perenne L. Plant Breeding 98, 149–155.
[2] Cooper J.P. (1959): Selection and population structure in Lolium.II: Genetic control of date of ear emergence. Heredity 13, 445–459.
[3] Falcinelli M. (1999): Temperate forage seed production. Journal of New Seeds 1, 37–66.
[4] Fang C., Aamlid T.S., Jørgensen Ø. and Rognli O.A. (2004): Phenotypic and genotypic variation in seed production traits within a full-sib family of meadow fescue. Plant Breeding 123, 241–246.
[5] Laidlaw A.S. (2005): The relationship between tiller appearance in spring and contribution to dry-matter yield in perennial ryegrass (Lolium perenne L.) cultivars differing in heading date. Grass and Forage Science 60, 200–209.
[6] Studer B., Jensen L.B., Hentrup S., Brazauskas G., Kölliker R. and Lübberstedt T. (2008): Genetic characterisation of seed yield and fertility traits in perennial ryegrass (Lolium perenne L.). Theoretical & Applied Genetics 117, 781–791.

DOI: 10.5507/vup.21.24459677.29

Better utilization of *Lolium perenne* in biorefineries based on its chemical composition

Yuhong Shi[1], Jörg-Michael Greef[1] and Katrin Kuka[1]

[1] Julius Kühn Institute (JKI), Federal Research Centre for Cultivated Plants, Institute for Crop and Soil Science, Bundesallee 58, 38116 Braunschweig, Germany

yuhong.shi@julius-kuehn.de

Abstract. The cutting date has a major effect on the quality and chemical composition of perennial ryegrass (*Lolium perenne*) biomass. The present study was conducted to find the impacts of cutting date on grass biomass qualities, in order to develop a better utilization strategy of *L. perenne* in biorefineries. The nutritive values of ten different *L. perenne* varieties, each cut at two different maturity stages, were measured using near-infrared spectroscopy (NIRS). We found that the cutting dates had essential effects on grass quality. Further work will focus on defining the cutting dates regarding specific grass utilizations.

Keywords: *Lolium perenne*, chemical composition, NIRS

1 Introduction

Perennial ryegrass (*Lolium perenne*) is one of the most valuable and widely spread forage crops in temperate grassland owing to its high yield and digestibility. The yield and quality of *L. perenne* highly depends on the growth stage and the variety it belongs to [4]. Swieter et al. [4] have shown in 2014 that the optimal cutting date of *L. perenne* for biogas production could be determined by monitoring and modelling the yield and quality dynamic of the grass.

The excess in biomass supply from grassland is occurring across some regions of the EU due to the increase of forage crop and concentrate feed [1]. Thus interest has been growing in developing alternative utilizations of grass biomass from grassland.

The aim of our study was to develop a strategy for better specific utilizations of *L. perenne* herbage in bioenergy production or as raw materials in biorefineries (e.g. ethanol, lactic acid, and unicellular protein), taking into account their chemical compositions depending on the variety-specific optimal cutting date. Due to the early stage of the project, which starts in August 2020, only the analysis of the first cut of one of 19 experimental sites is discussed here.

2 Material and Methods

A field experiment was established in Völkenrode (52°28´N, 10°40´E) in Germany in 2016, with ten *L. perenne* varieties with different maturing rates. The experiment was set-up following a randomized block design with three replications. For each variety, two distinct harvest times were carried out in the first cut in 2017 according to the actual maturity of particular reference varieties (i.e. when reference varieties reached BBCH stage 51). At each cutting date, the growth stage (recorded in BBCH-scale; [2]) and DM yield were recorded. Different nutritive values including crude protein (CP), water soluble carbohydrates (WSC), crude fibre (CF), neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), and enzyme soluble organic matter (ELOM; [3]) were measured using a Foss NIRSystem 5000 spectrometer (Foss GmbH, Hamburg, Germany). The NIRS-measurements began in November 2020. The content of cellulose, hemicellulose and lignin were derived from NDF, ADF and ADL (i.e. cellulose = ADF – ADL; hemicellulose = NDF – ADF; lignin = ADL). Proportion of CF, CP, WSC of their total and proportion of WSC, cellulose, hemicellulose, and lignin of their total were calculated. Change rates were then calculated through dividing change of particular proportion by increase in BBCH value. The DM yield was determined through samples dried at 105 °C for 24 h. The ground herbage samples for NIRS-measurement were dried at 60 °C for 48 h.

3 Results and Discussion

Before all varieties reached BBCH-51, the proportion of CF increased with grass maturity, while the proportion of CP and WSC in the plant biomass decreased along with ELOM values (Fig. 1a). This is due to the increase in cell wall components which results in a decrease in plant digestibility. As a result, varieties with higher increase rate in CF proportion showed higher decrease in ELOM value. Similar changes in these nutritive values were also found in plant after BBCH-51 (Fig. 1b). However, the proportion of CF increased more rapidly in nearly all varieties compared to herbage harvested before BBCH-51. This rapid increase in structural components was likely caused by the stem elongation as plants entered the reproductive phase. Notably, the behaviour of varieties was totally different before and after the grasses reached growth stage BBCH-51 (Fig.1a & 1b). A similar trend of changes was observed in the compositions of carbohydrates. While the proportions of cellulose, hemicellulose and lignin increased with grass maturity, the proportion of WSC decreased (Fig. 1c & 1d). Almost all varieties had higher increase rate in cellulose proportion after BBCH-51 than before, except varieties 8 and 2. We confirmed that the chemical composition in *L. perenne* changes with plant maturity and that the rate of change varies among varieties, implicating essential influences of cutting date on the quality of *L. perenne*. Our

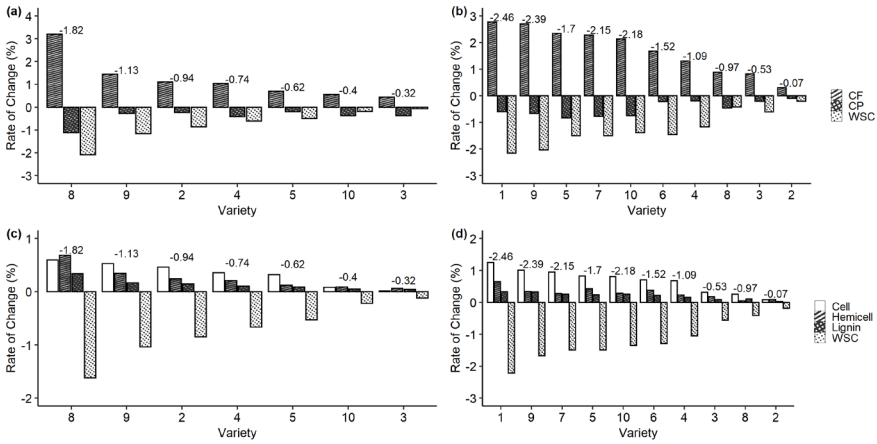


Fig. 1. The change rate of CF, CP, and WSC (a) before and (b) after BBCH-51, and of cellulose, hemicellulose, lignin and WSC (c) before and (d) after BBCH-51 for different varieties of *L. perenne*. Positive values indicate an increase in proportion, while negative values indicate decrease in proportion. The numbers above each bar stand for the change rate of ELOM.

future work will analyse the chemical compositions based on dry matter yield and take into account the requirements of specific grass utilizations.

Acknowledgments

We would like to thank the FNR for the funding and all grass breeders as well as the BSA for carrying out and helping with the grassland experiments.

References

[1] Leclère D., Valin H., Frank S. and Havlik P. (2016): Assessing the land use change effects of using EU grassland for biofuel production. Task 4b of tender ENER/C1/2013–412.

[2] Meier U., Bleiholder H., Buhr L., Feller C., Hack H., Hess M., … Zwerger P. (2009): The BBCH system to coding the phenological growth stages of plants – history and publications. *Journal of Cultivated Plants* 61, 41–52.

[3] Sundstöl F., Kossila V., Theander O. and Thomsen K.V. (1978): Evaluation of the feeding value of straw: a comparison of laboratory methods in the Nordic countries. *Acta Agriculturae Scandinavica* 28, 10–16.

[4] Swieter A., Moenickes S., Ohnmacht B., Greef J.-M. and Feuerstein U. (2014): Monitoring, analysis and modeling of yield and quality dynamics of *Lolium perenne* varieties for biogas production. In: Sokolović, D., Huyghe, C. and Radović, J. (eds) *Quantitative Traits Breeding for Multifunctional Grasslands and* Turf, Springer, Dordrecht, Netherlands, pp. 325–330.

Session 4: “Minor” and “new” species – solution for future challenges

 DOI: 10.5507/vup.21.24459677.30

Shall we add some flowers or even add some weeds – will this be the solution for business of seeds?

Fred Eickmeyer

ESKUSA GmbH, Bogener Str. 24, D-94365 Parkstetten, Germany

eickmeyer@t-online.de

Abstract. Implementation of European law might have strong influences on future species composition of perennial grassland and forage production. For different reasons it might be necessary to (re)add additional species to forage production fields. Multiplication of wild herbs for municipal areas has already been started as a consequence of the convention on biological diversity with economic success for the companies that started to produce seed in time. For the same reasons it can be expected that agricultural production fields will have to contribute in the frame of their possibilities to deliver biodiversity in future. Wild herbs in intensive permanent grassland have decreased strongly. There are reasons to predict that especially the permanent grassland will not only be worth but might be forced legally to contribute to biological diversity in a more intensive manner than it did during the last decades. The functional contributions of wild herbs and minor forage species to grassland in terms of animal health, food quality, climate change adaptability and biological diversity are discussed. Breeding and seed multiplication of wild herb species are presented as an option. Opportunities for breeders, seed multipliers and seed merchants are outlined.

Keywords: Pasture herbs, Convention on biological diversity, Permanent grassland composition, Biodiversity, Functional species

1 Grassland Development and Situation for Herbs

Around 1970 the first cut in pastures was carried out mid of June for hay production in north Germany when the weather conditions were adequate. Silage was by far not so present as it is today. By mid of June several of the herbs and grasses existing in pastures have spread at least a part of their seeds and founded the next generation – a natural reseeding of the sward took place.

During the last decades, a strong focus of forage production was laid on energy content of silage to serve the necessities of high-performance milk and beef production. Energy content, water soluble carbohydrates and protein were the most relevant characters to describe the quality of the silage. In order to harvest young grasses, the first silage cut was brought forward about six to seven weeks

to end of April / early May to deliver highest energy content. Pasture herbs have no chance to spread their seeds until that date.

Four to six cuts are reached in some regions with the help of high (mineral) fertilization input and regular reseeding of grass seeds. Fertilization favors grasses in comparison to herbs and while the grasses are reseeded to keep dense swards, the herbs are not.

This management led to a decrease of valuable herbs in grassland and was accepted for decades as the best practice management of grassland. For protein content reasons a few legume species like white clover was selected for pastures, while red clover and alfalfa were selected for lays. Lots of further legumes such as *Galega*, *Onobrychis* and *Lotus*-species still wait for their chance to be included in seed mixtures [1]. For pasture herbs, selection was never done with a few exceptions such as chicory (*Cichorium intybus*) and plantain (*Plantago lanceolata*) in overseas [2].

Farmers know how difficult it is to keep a well-balanced relation between grasses and clovers in their forage production. It is even more challenging to keep additional herbs in a balance with grasses and clovers. Therefore there are several reasons to stick to pure grass/clover stands and to avoid complication of grasslands management by adding more species.

2 Time to Change Composition of Permanent Grassland?

There are also several reasons and necessities that speak for a (re)development of grassland into a species rich habitat that does not only produce feed for livestock but at the same time delivers additional ecosystem services. Legal changes and climate change are factors that put a strong pressure on alternative grassland management and will have consequences on future grassland composition.

Legal pressure and the European biodiversity strategy

After the text of the Convention on Biological Diversity [3] was adopted in 1992 in Nairobi, within a year, it had received 168 signatures and entered into force in December 1993. In accordance with Article 6 of the Convention, parties have to develop national biodiversity strategies or action plans. Together with the Council Directive 92/43/EEC of 21 May 1992 [4] on the conservation of natural habitats and of wild fauna and flora, a strong legal basis was formed to implement biodiversity aspects also in agricultural decisions.

These legal specifications – together with some scientific preparations [5] [6] – were an initial step and formed the basis for controlled seed multiplication of wild herbs for use in mixtures in municipal green spaces. A new market was created by the implementation of this law on a national basis. In the meantime, there was an increasing demand for local mixtures of wild plants for local author-

ity districts with good income for seed merchants and seed multiplying farmers, controlled by means of a reasonable certification system [7] [8].

The EU's biodiversity strategy [9] for 2030 – a core part of the European Green Deal – is an ambitious long-term plan to protect nature and reverse the degradation of ecosystems. The strategy aims to put Europe's biodiversity on a path to recovery by 2030, and contains specific actions and commitments.

It is obvious that further steps to implement this biodiversity strategy will have impacts on agriculture in general and on the future management and structure of permanent grassland within Europe. Strategies of ground water protection, fertilizer reduction and insect protection will put further pressure on change of grassland management and species composition.

Climate change

The dry summers in the years 2018–2020 showed painfully that even in the maritime areas of Europe, the perennial ryegrass-based swards were very sensitive to drought and failed to produce enough forage in many regions. Farmers think over using more drought tolerant grasses like cocksfoot, tall fescue and tall oatgrass. These grasses have a deeper rooting system and could be companioned by drought tolerant legumes like alfalfa, *Onobrychis*, *Melilotus, Lotus* and deep rooting herbs like *Cichorium, Taraxacum, Plantago, Sanguisorba, Pimpinella* and other species.

Ongoing climate change, rising summer temperatures with prolonged periods of drought will favor deep rooting plants. Even if such mixed swards will not be able to deliver the highest amount of energy, they are more drought tolerant and produce at least a certain amount of forage under drought conditions.

3 Functional Herb Species

It is for sure that legal and climate changes will force farmers to change their grassland management and it might be one consequence to add more herb species to a so far predominantly grass-based sward.

From the energy-content point of view, forage herbs might have a negative image with the farmers; however there are several beneficial functions of herbs that are still well known in human folk-medicine [10] but are forgotten by practical farmers in animal feeding. About 100 years ago, several herbs were regarded as valuable in permanent grassland because they kept the animals healthy in a natural way by voluntary intake. In terms of animal health, milk- and beef quality functional herbs might have a rediscovery. Terms such as "Alpenmilch" or "Hay-cheese" imply herb and wildflower rich pastures in an intact ecosystem with small family owned farms. They are used successfully in marketing beef- or

milk-products today. But there is more behind it than marketing. The following examples describe animal health and - productivity functions of herbs:

Palatability

Spicy herbs [11] like caraway (*Carum carvi*), thyme (*Thymus pulegioides*), chives (*Allium schoenoprasum*) improve the palatability of the forage, increase the voluntary intake and have positive effects on digestion. Aetheric oils or sulfur-containing substances are responsible for the palatability effects. In addition, these spices have antimicrobial activities and anti-inflammatory effects.

Bitter tasting substances in *Cichorium intybus, Taraxacum officinale, Centaurium erythraea, Arnica, Primula, Cardamine* and many other species stimulate salivation and improve digestion [10].

Rumen health and productivity

Lippert [12] described already in 1953 that yeasts (maybe also fungi and bacteria) existing in the flowers of Lamiaceae refresh the composition of the rumen microbiome to keep it on a high-performance level. Examples can be found in the following species and genera: *Salvia pratensis, Ajuga repens, Glechoma, Prunella, Scutellaria, Thymus, Mentha, Lamium, Stachys, Origanum, Galeopsis and Melissa.*

Sainfoin, Lotus and a few other legume species contain condensed tannins and other polyphenolic ingredients that reduce foam production in the rumen and act for a better protein availability [11].

Milk and beef quality

Legumes play an important role in fatty acid composition of milk and beef [1]. Herbs in general deliver higher mineral amounts than grasses [13]. These minerals can be found in beef and milk.

The genera of *Alchemilla, Trigonella, Galega, Cnicus Foeniculum, Pimpinella, Carum, Silybum, Medicago, Melissa* and *Althaea* are recommended for stimulation of lactation in human [14]. Even if there is a big difference between the digestion system of humans and cattle there is no doubt that not at least some of the above mentioned genera are also lactation stimulants in livestock.

Intestinal worms

Tannine-rich species play an important role to control intestinal worms [15]. Genera like *Artemisia, Chenopodium, Tanacetum, Allium, Polygonum, Onobrychis, Lotus* are regarded as anthelminthic.

The above-mentioned functional effects are only a small part of functions that wild herbs can deliver to livestock feeding and animal health. Some of these herbs are traditionally used in veterinary medicine and there is an increasing interest in treating livestock diseases on a phytomedicinal basis.

Because of lacking persistence not all health-functional herbs are suitable to be included in a grassland community. There are approaches to use these functional herbs as a "field-border-pharmacy" in a special species mixture for grazing cattle, sheep or horses at the side of the fields as a voluntary intake choice.

Biological diversity and insect protection

In several areas in Europe, landscape aesthetics plays an important role for tourism. Adding flowers to the grass-based green pastures can be very attractive to tourists and will give the farmers another image.

Biological diversity is gift for humans per se. We have learned that a diverse nature is the best buffer against many mistakes of human activities.

Adding herbs to grassland together with a delayed first cut offer hideaways for insect larvae and adults. Especially umbelliferous herbs play a very important role in this regard.

4 Opportunities

Forage breeders are used to work with several different species of monocots and dicots. The intensity of the breeding program is correlated with the economic importance of the species. For wild herbs, it might not be necessary to select very intensively because one contribution of herbs to a grassland community is their wide within-species genetic variation.

Nearly all of the wild herb species are not included in the list of species and therefore neither have to fulfill requirements of the value for cultivation and use (VCU) nor have they to be distinct, uniform and stable (DUS). That allows multiplication of collected seed from local wild populations without undergoing the traditional seed marketing act. The breeders and seed multipliers already have the equipment to harvest and to process most of the herb seeds. This means that, within a very short time seeds of wild herbs could be marketed. Special harvest equipment might be necessary to harvest for example *Taraxacum, Leontodon* or *Arnica*-seeds. At ESKUSA, a company that aims at seed production of special crops, dandelion seeds are harvested with a combination of a rotating brush with a vacuum sucker. Other species are only brushed for seed production because the seed development is very asynchronous.

The multiplied seed can be added to forage seed mixtures or used as pure seeds to reseed existing pastures. Besides this, the multiplied seed can be used also for municipal areas and, last but not least, there is an increasing number of

small online stores selling small (very expensive) portions of wild herb seeds to gardeners, beekeepers and other insect friends. A few kilograms of wild herb seeds often have the same price as a few tons of a conventional grass variety.

Multiplication of ecotypes of grass and clover species and wild herbs can be a very profitable business. Often starting with a niche, wild herb seed production can become a separate and economically interesting business model for breeders, seed multipliers and seed merchants.

As a long term perspective also, the wild herbs can be bred for several characters e.g. competing ability, synchronous development to companion partners, synchronous seed ripening, functional ingredients and last but not least biomass and forage yield. Selection is an option, however herb species can also be multiplied without prior selection.

References

[1] Vaz Patto C. (2016): Legume Perspectives. For animals, humans and environment - Legumes in ruminant feeding. The journal of the International Legume Society, 12

[2] Labreveux M., Sanderson M.A., Hall M.H. (2006): Forage Chicory and Plantain: Nutritive Value of Herbage at Variable Grazing Frequencies and Intensities. Agron. J. 98:231–237. https://doi:10.2134/agronj2005-0012

[3] CBD-Homepage, https://www.cbd.int/convention, last accessed 2021/13/06.

[4] EUR-LEX-Homepage,https://eur-lex.europa.eu/legalcontent/EN/TXT/?uri=celex%3A31992L0043, last accessed 2021/13/06

[5] Knevel, I.C., Bekker, R.M, Kunzmann, D., Stadler, M. & Thompson, K. (2005): The LEDA Traitbase Collecting and Measuring Standards of Life history Traits of the NW European Flora. Published by the University of Groningen, Groningen, The Netherlands. Available: http://www.LEDATraitbase.org.

[6] Kleyer,M., Bekker R.M., Knevel I.C., Bakker J.P., Thompson K., Sonnenschein,M., Poschlod P., van Groenendael J.M., Klimes L., Klimesová J., Klotz S., Rusch G.M., Hermy M., Adriaens D., Boedeltje G., Bossuyt B., Dannemann A., Endels P., Götzenberger L., Hodgson J.G., Jackel A-K., Kühn I, Kunzmann D., Ozinga W.A., Römermann C., Stadler M., Schlegelmilch J., Steendam H.J., Tackenberg O., Wilmann B., Cornelissen J.H.C., Eriksson O., Garnier E. and Peco B. (2008): The LEDA Traitbase: A database of life-history traits of the Northwest European flora. Journal of Ecology. 96. 1266 – 1274. 10.1111/j.1365-2745.2008.01430.x.

[7] Prasse, R., Kunzmann, D. & R. Schröder (2011): Forschungsprojekt Regiosaatgut. Grundlagen für bundeseinheitliche Regionalisierung der Wildpflanzenproduktion (Saat- und Pflanzgut). Natur in Nordrhein-Westfalen 2/11, p. 30–32.

[8] Forschungsgesellschaft Landschaftsentwicklung Landschaftsbau e.V. – FLL [Hrsg.] (2014): Empfehlungen für Begrünungen mit gebietseigenem Saatgut. Bonn, 1.Ausgabe: 123S.

[9] EC-Homepage, https://ec.europa.eu/environment/strategy/biodiversity-strategy-2030_de, last accessed 2021/13/06.[10]Van Wyk, B.-E., Wink C., Wink M. (2004): Handbuch der Arzneipflanzen. Wissenschaftliche Verlagsgesellschaft, Stuttgart. ISBN 3-8047-2069-2.

[10] Van Wyk, B.-E., Wink C., Wink M. (2004): Handbuch der Arzneipflanzen. Wissenschaft-liche Verlagsgesellschaft, Stuttgart. ISBN 3-8047-2069-2.

[11] Häring, D., Scharenberg, A., Heckendorf, F., Dohme, F., Lüscher, A., Maurer, V., Suter, D., Hertzberg, H. (2008): Tanniferous forage plants: Agronomic performance, palatability and efficacy against parasitic nematodes in sheep. Renewable Agriculture and Food Systems: 23, 19–29.

[12] Lippert, F. (1953): Vom Nutzen der Kräuter im Landbau. Schriftenreihe "Lebendige Erde", Forschungsring für Biologisch-Dynamische Wirtschaftsweise, Darmstadt ISBN 3-921536-05

[13] Pirhofer-Walzl, K., Søegaard, K., Høgh-Jensen, H., Eriksen, J., Sanderson, M.A., Rasmussen, J., Rasmussen, J. (2011): Forage herbs improve mineral composition of grassland herbage. Grass and Forage Science 66(3):415 – 423. https://doi.org/10.1111/j.1365-2494.2011.00799.x

[14] Calzetta, L., Pistocchini E., Leo A., Roncada P., Ludovica Ritondo B., Palma E., di Cave D., Britti D. (2020): Anthelminthic medicinal plants in veterinary ethnopharmacology: A network meta-analysis following the PRISMA-P and PROSPERO recommendations. https://doi.org/10.1016/j.heliyon.2020.e03256.

[15] Kingsbury S. (2012): Herbs for lactation. Journal of the American Herbalists Guild, 11 (1), 41–46.

DOI: 10.5507/vup.21.24459677.31

Potential for developing improved rhizobium inoculants and lucerne varieties for efficient symbiosis in sustainable agriculture

Åshild Ergon[1], Yuan Gao[2], Tina Schmaucks[1] and Åsa Frostegård[2]

[1] Norwegian University of Life Sciences, Faculty of Biosciences, Ås, Norway
[2] Norwegian University of Life Sciences, Faculty of Chemistry, Biotechnology and Food Science, Ås, Norway

ashild.ergon@nmbu.no

Abstract. The N-fixing rhizobial strain *Ensifer meliloti* 1021 (E.1021) has the ability to consume N_2O, providing an opportunity of greenhouse gas mitigation. We found that two commonly used commercial rhizobium inoculants neither consumed nor produced N_2O and had a lower symbiotic efficiency than E.1021. Lucerne varieties also varied in their symbiotic efficiency, irrespective of inoculum type. The interaction between rhizobia and agricultural crops can be better utilized through plant breeding and development of multifunctional inoculants.

Keywords: lucerne, nitrous oxide, rhizobia.

1 Introduction

Plant-rhizobium interactions often have a degree of specificity, resulting in specific combinations of genotypes having greater symbiotic efficiency than others. This could be utilized both in plant breeding and in development of inoculants. Moreover, rhizobial strains vary in their ability to catalyze the different steps of the reduction of NO_3^- to N_2 (NO_3^-→NO_2^-→NO→N_2O→N_2). Strains that lack the last step (N_2O reduction) will act as sources for N_2O while strains that are able to carry out this step can act as N_2O sinks [1]. Using N_2O-reducing strains as rhizobium inoculants could therefore be a strategy to mitigate agricultural N_2O emissions. We here 1) assessed the ability of two commercially available inoculants to consume N_2O in a gas kinetics experiments and 2) characterized variation in the symbiotic efficiency of various combinations of rhizobial strains and lucerne (*Medicago sativa*) varieties.

2 Material and Methods

Nitragin Gold from Bayer, the lucerne inoculant from Inocula scandinavica, and the N_2O-reducing strains *Ensifer meliloti* 1021 (E.1021), AC50a, AC50e and AC52a2 [1, 2] were used in combination with a diverse set of lucerne varieties originating from Norway (Lavo, Live, Lotte, Ludvig), France (Luzelle, Verdor), Serbia (Banat VS) and the USA (5246). For analysis of gas kinetics, precultures were raised in yeast mannitol broth (YMB, pH 7). One ml preculture with an OD_{600} of 1.0–1.6 was added to incubation vials (n=3 per inoculant) with 1 mM KNO_3. The headspace consisted of He with 1 % (vol.) O_2 and 40 µmol N_2O $flask^{-1}$. At 20.8 h, KNO_2 was added to a concentration of 0.5 mM. Flasks were incubated at 28 °C, and the headspace gases were monitored by a robotized gas analysis system [3].

Two inoculation experiments were conducted; exp. A with E.1021, the two commercial inoculants and all lucerne varieties, and exp. B with the four N_2O-reducing strains of *E. meliloti* and all lucerne varieties except Lavo and Lotte. The inoculants were raised in YMB for two days prior to inoculation, at which time OD_{600} was 0.50–0.65 in exp. A and around 1 in exp. B. Lucerne seeds were surface sterilized, scarified and germinated on filter paper, transferred to pots with vermiculite and perlite (75/25 v/v) together with 1 ml inoculum per seed (3 plants per pot). Pots were organized in a split-plot design with inoculant as the main plot factor and variety as the subplot factor, all replicated in 7 randomized complete blocks. Inoculated plants were regularly given a fertilizer without N, while uninoculated positive controls were given the same fertilizer supplemented with N. Symbiotic efficiency was expressed as the ratio of the dry weight of inoculated plants of a variety on the average of the positive controls from the same variety at the end of the experiment and subjected to analysis of variance in R, using the function *lmer* in the package *lmerTest*.

3 Results and Discussion

Gas kinetic analysis of the two commercial inoculants showed that they do not reduce N_2O when O_2 is depleted, but rather reduce NO_2^- to NO, without reducing NO to N_2O (Fig. 1B). Thus, they are neither producers nor consumers of N_2O. Exp. A showed that E.1021, in addition to being able to consume N_2O (Fig. 1A), had higher symbiotic efficiency than the two commercial inoculants when infecting lucerne (Fig. 2A). Some legume crops must be inoculated with compatible rhizobia prior to sowing; this is for example the advised practice for lucerne cultivation in Norway. Hence, the potential for developing inoculants that contribute to reduced N_2O emissions and at the same time are more efficient should be explored further. The N_2O-consuming strains AC50a, AC50e, AC52a2 were not compatible with any of the varieties, even if they are compatible with other varieties than those studied here [2].

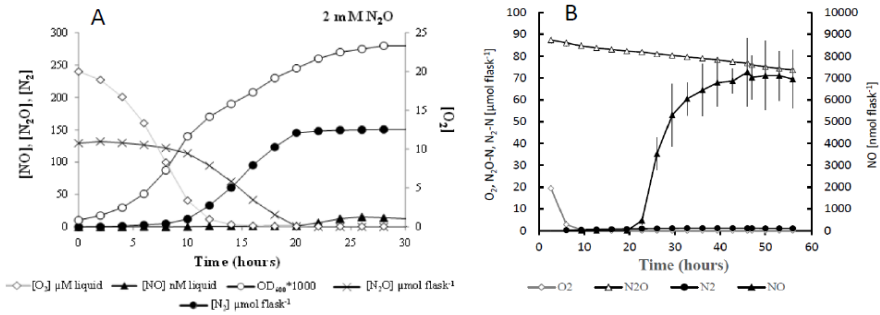


Fig. 1. Gas kinetics in A) E.1021 (reproduced from [1]) and B) lucerne inoculum from Inocula scandinavica (average of 3 replicates ± s.d., Nitragin Gold showed a similar pattern). The slight reduction in N_2O reflects a dilution effect from gas sampling, as there was no increase in N_2.

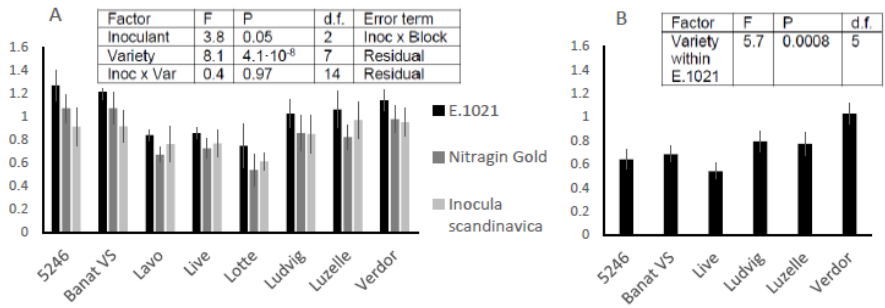


Fig. 2. Symbiotic efficiency of lucerne varieties and inoculum types in two experiments (A and B), measured as the ratio of the dry weight of inoculated plants on average dry weight of non-inoculated N-fertilized plants of the same variety (averages ± S.E.). Strain AC50a, AC50e, AC52a2 did not infect any variety in exp. B, hence results are shown for E.1021 only. Results from analysis of variance are shown in inserted tables.

Lucerne varieties also differed in their symbiotic efficiency (Fig. 2). When inoculated with E.1021, some of the varieties ranged in the same order in both experiments (Live, Ludvig, Luzelle and Verdor) while others, for unknown reasons, ranged differently (5246 and Banat VS). The observed variation in symbiotic efficiency among lucerne varieties suggests that this trait could be improved through breeding.

It is not known whether the commercial inoculants consist of a mixture of strains or not, but the lack of statistical interaction between inoculum and variety (Fig. 2A) suggests that the level of specificity is low, which would simplify the development of both inoculants and varieties for sustainable agriculture.

Acknowledgements

This study was funded by Norwegian Research Council grant number 283429. We are grateful to Bernadette Julier, Helga Amdahl and Kristin Gylstrøm for providing seeds.

References

[1] Bueno, E., et al. (2015): Anoxic growth of *Ensifer meliloti* 1021 by N2O reduction, a potential mitigation strategy. Front. Microbiol. 1(6).

[2] Woliy, K.J(2019).: Rhizobia in climate-smart agriculture - N_2 fixation effectiveness and N_2O reduction capacity of phylogenetically diverse *Bradyrhizobium* and *Ensifer* strains. PhD thesis, Norwegian University of Life Sciences. 2019:92, ISSN 1894-6402.

[3] Gao, Y., et al (2021).: Competition for electrons favors N_2O reduction in denitrifying *Bradyrhizobium* isolates. Environ. Microbiol. 23: 2244–2259.

DOI: 10.5507/vup.21.24459677.32

New approaches to the establishment of mixed cultures of alfalfa with grasses

Tomas Vymyslicky[1], Danilia Knotova[1], Jiri Skladanka[2], Pavel Knot[2] and Martin Brtnicky[2]

[1] Agricultural Research, Ltd., Zahradní 1, 664 41 Troubsko, Czech Republic
[2] Faculty of Agronomy, Mendel University, Zemědělská 1, 613 00, Brno, Czech Republic

vymyslicky@vupt.cz

Abstract. As a consequence of climatic change, alfalfa is becoming the most important forage crop among legume species in the conditions of the Czech Republic. In this study, we focused on mixed cultivation of alfalfa with grasses grown on alternate rows. First results showed advantages of mixed culture compared to pure alfalfa stands. Results from the first harvest year let foresee a significant potential of mixed culture for wider use than only organic agriculture in the context of climatic change.

Keywords: Intercropping, Row density, Hay yields.

1 Introduction

Climatic change is significantly affecting central Europe [2]. Alfalfa is becoming the most important forage crop among legume species in the conditions of the Czech Republic, while in the past it was the red clover [4]. Agrotechnology of pure stand cultivation is well established and widely used. Conventional technologies of legume-grass mixtures including alfalfa have also been widely studied and novel combinations of species have been examined in the context of foreseen climatic change [3]. Mixed culture in the form of different species grown in alternate rows is a new approach in our conditions. First results have shown its advantages in dry periods, especially because of limited inter species competition [3].

The main aim of our research was to test different combinations of alfalfa and grasses grown at several row densities and to evaluate weed infestation and hay yields as compared to pure alfalfa stand.

2 Material and Methods

Field trial with randomised design was sown on arable land in April 2019, at locality Troubsko. The soil was deeply ploughed in the autumn and the seed bed was thoroughly prepared in the early spring. Special sowing machine STP 300 was used. Sowing depth was 1,5 cm and spacing between rows was 15 cm. Plot size was 3x10 metres, including 20 rows. A rhizobium inoculant was applied on one half of the trial whereas the second half was left free of application to serve as control. Alfalfa and grasses were sown in alternate rows with same sowing rates for grasses and alfalfa, *i.e.* 20 kg ha^{-1}. See Table 1 for more details.

Three experimental factors were studied: grass species, number of rows of grasses and the use or not of rhizobium inoculant. Pure alfalfa stand was used as control plot.

Cover values of alfalfa, grass and weeds were visually assessed at four dates (4 May 2020, 25 June 2020, 11 August 2020 and 5 October 2020) in the first harvest year. Box and whisker plots were used to visualize the differences in Figure 1.

Harvest was performed at the same dates as visual assessment of the stands. Hay yield was weighted separately for each species and compared to pure alfalfa stand at the same days. ANOVA and Tukey post hoc test were used for the statistical analyses.

Table 1. Alfalfa and grass cultivars used in field trial

Alfalfa	Grass	Numbers of rows alfalfa : grass
cv. Jarka	Orchard grass cv. Harvestar	I) 1 row : 1 row; II) 1 row : 2 rows; III) 1 row : 3 rows
cv. Jarka	*Festulolium* cv. Fedoro (loloid hybrid)	I) 1 row : 1 row; II) 1 row : 2 rows; III) 1 row : 3 rows
cv. Jarka	*Festulolium* cv. Hykor (festucoid hybrid)	I) 1 row : 1 row; II) 1 row : 2 rows; III) 1 row : 3 rows

3 Results and Discussion

The row cover (row density) of cultivars was significantly influenced by the grass species. The rows of the two different *Festulolium* hybrids were significantly denser and included a smaller proportion of weeds than those of alfalfa and orchard grass. The densities of alfalfa and grass rows were comparable when *Festulolium* was used, but there was a higher share of weeds with loloid *Festulolium* due its slower ontogenetical development. Festucoid type of *Festulolium* showed better performance than both orchard grass and loloid type of *Festulolium*. See Figure 1 for details.

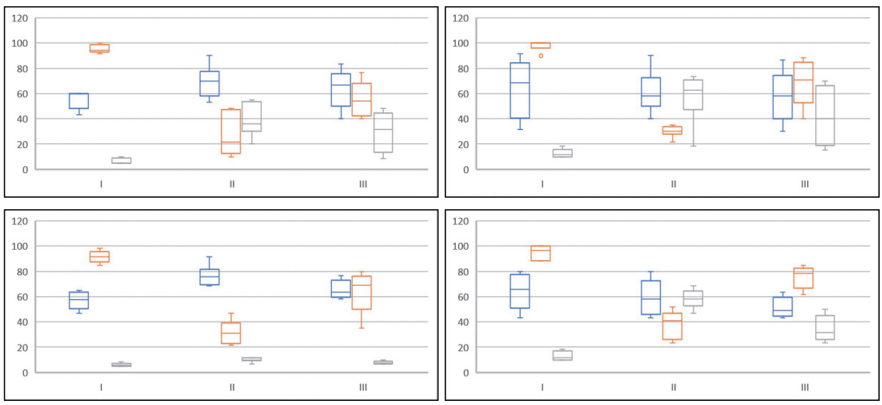


Fig.1. The influence of grass species, (I) *Festulolium* Fedoro, (II) Orchard grass, (III) *Festulolium* Hykor, on the rate of row cover (row density) by cultivars of alfalfa (blue), grass (red) and weeds (grey) at for dates in the first harvest year based on visual assessment. Row density values are based on four terms of visual assessment (4.5., 25.6., 11.8. and 5.10.).

The number of grass rows had no significant effect on the average row density of both alfalfa and grass and the row densities of alfalfa and grasses were comparable. See Figure 2 for details.

The rhizobium inoculant used in the establishment of trial had a slightly significant positive effect on the biomass production. Details are given in Figure 2.

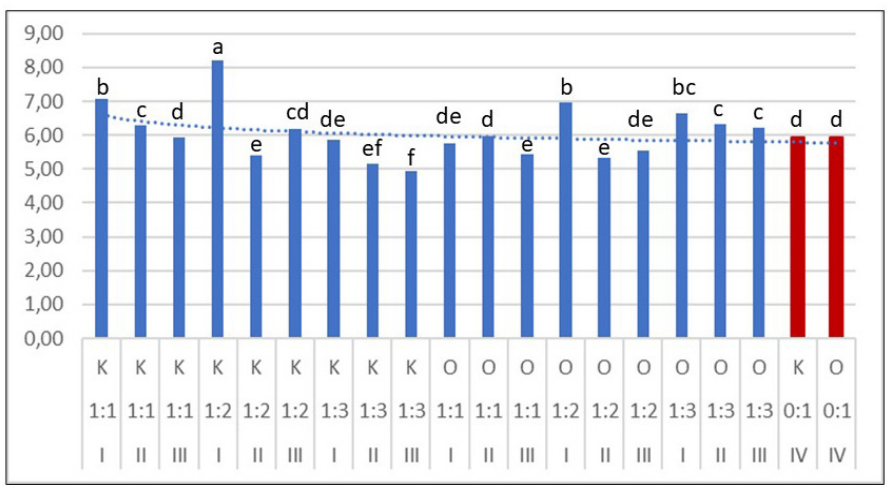


Fig. 2. Hay yields (t.ha^{-1}) cumulated across year of the different alfalfa – grass mixtures (blue) compared to alfalfa pure stand (red), (I) *Festulolium* Fedoro, (II) Orchard grass, (III) *Festulolium* Hykor, (K) no inoculant applied, (O) inoculant applied. Lowercase letters above the columns indicate statistically significant differences in yield.

Figure 2 also shows the average hay yields of the different alfalfa - grass mixtures compared to that of alfalfa monoculture. We can see that six over 18 of the mixtures exceeded the alfalfa pure stand.

Results obtained for the first harvest yield showed a significant potential of mixed culture for wider use in agriculture under the conditions of climatic change. Based on these results, the *Festulolium* loloid hybrid Fedoro could be recommended. Positive results of *Festulolium* hybrids compared to other grasses are well known [1].

Acknowledgement

Research leading to these results received funding from the Technological Agency of the Czech Republic, project no. TH04030258.

References

[1] Adamovich A. and Adamovicha O. (2003): Productivity and forage quality of Festulolium/legume mixed swards in response to cutting frequency. In Optimal forage systems for animal production and the environment. Proceedings of the 12th Symposium of the European Grassland Federation, Pleven, Bulgaria, 26–28 May 2003 (pp. 453–456).

[2] Ballester J., Rodó X. and Giorgi F. (2010): Future changes in central Europe heat waves expected to mostly follow summer mean warming. Climate Dynamics, 35(7–8), 1191–1205.

[3] Cox S., Peel M. D., Creech J. E., Waldron B. L., Eun J.-S., Zobell D. R., Miller R. L. and Snyder D. L. (2017): Forage Production of Grass–Legume Binary Mixtures on Intermountain Western USA Irrigated Pastures. Crop Science, 57: 1742–1753.

[4] Czech Statistical Office (2021): Areas Under Crops. Available at https://vdb.czso.cz (15. 4. 2021)

Festulolium Workshop

 DOI: 10.5507/vup.21.24459677.33

Development of KASPar markers in *Festulolium* hybrids

Sabrina Delaunay[1], Philippe Barre[1], Sebastian Blugeon[1], Camille Gréard[2], David Kopecky[3], Marc Ghesquière[1]

[1] INRAe/URP3F – Lusignan 86600, France
[2] Jouffray-Drillaud – St-Sauvant 86600 France
[3] Institute of Experimental Botany, Šlechtitelů 31, CZ-78371 Olomouc, Czech Republic

Marc.Ghesquiere@inrae.fr

Abstract. Festulolium are interspecific hybrids between ryegrass and fescue species whose genomes in combination may to varying extents, genetically recombine. From a breeding perspective, both in amphiploid hybrids and their back-cross derivatives, it is valuable to determine the extent of any interspecific genome recombination and consequent allelic substitution of one species by another at any one homeologous chromosome locus. Towards this objective, the genotype distribution at 6 SNP loci located on distinct linkage groups was studied using KASPar technology in 3 contrasting *L. multiflorum* x *F. glaucescens* populations (2n=4x=28). From first generation hybrids and the amphiploid cv Lueur, the results confirm the predominant disomic inheritance of Festulolium from *F. glaucescens*. Thus, the average interspecific heterozygosity in the cv Lueur remains close (0.42) to that of F1 hybrids while it decreased to 0.13, on average, in a population in which only one copy of the *Festuca* genome was retained following one backcross into ryegrass (BC1). Furthermore, significant differences of frequency were evidenced across SNPs, from 0.08 to 0.26, suggesting possible antagonistic genome selection within a segregating Festulolium population. A next step will be to identify among the 295 specific SNPs recently detected, those allowing the most accurate estimate of allele frequency in all sources of Festulolium.

Keywords: Molecular markers, Festulolium hybrids, Introgression rate, *Lolium multiflorum*, *Festuca glaucescens*

1 Introduction

Genotyping is a key element for further progress in plant breeding, particularly in tetraploid interspecific hybrids such as Festulolium. Gene distribution in hybrid progeny will depend on the extent of homologous vs homoelogous chromosome pairing from the parental species. Homologous preferential chromosome pairing of the genomes of the parental species will leading to disomic inheritance (e.g in *L. multiflorum* x *F. glaucescens*). In contrast, due to their genome affinities, amphiploid hybrids like *L. multiflorum* x *F. pratensis* may demonstrate homoeol-

ogous chromosome pairing and interspecific recombination leading to a quite different more tetrasomic genotypic distribution. So far, mostly in Festulolium amphiploid research, chromosomal painting (GISH) has been used to estimate interspecific heterozygosity but this is not compatible with requirements for high-throughput technologies necessary for plant breeding [2]. On the other hand, the development of molecular markers (DART) as an alternative, may lead to inaccurate estimates of allele frequency due to dominant inheritance [3]. To better enhance large scale genotyping in Festulolium, a search for homeologous SNP markers has been undertaken which we herein report the first genotyping of 3 contrasted Festulolium populations at 6 SNPs by using the KASPar technology [1].

2 Material and Methods

Six *L. multiflorum* vs *F. glaucescens* specific SNPs were sampled from a library of 295 SNPs recently identified in the *Festuca-Lolium* complex [this proceedings]. The KASPar genotyping was then applied to 3 contrasted populations resulting from *L. multiflorum* X *F. glaucescens* (2n = 4x = 28): i) 25 F1 hybrids, all expected of *Duplex* heterozygous genotype (*LLFF*) in respect with the substitution of two alleles of the *Lolium* genome (*L*) by two homeoalleles of the *Festuca* genome (*F*); ii) 60 individuals of the amphiploid cv Lueur, in which the distribution between *Simplex* (*LLLF*) and *Duplex* is expected to be more balanced and, iii) 59 individuals from one generation of backcrossing (BC1) into *L. multiflorum*, in which *Simplex* is expected to be the largest genotypic class. Furthermore, artificial *Simplex*, *Duplex* and *Triplex* (*LFFF*) controls were added from mixtures of pure DNA of each parent species in according genome ratio. Each individual in the 3 populations was visually scored as *Simplex* vs *Duplex* in addition to homozygous *LLLL* when no *Festuca* allele could be detected. Then, the observed genotype distribution was tested against an equilibrium assumption using X^2 statistics. The mean frequency of the *Festuca* allele in each population was estimated accordingly and compared between populations and between SNPs.

3 Results and Discussion

The scoring into the 5-genotype classes expected at a SNP locus within a tetraploid population is exemplified in figure 1. The genotype distribution and according estimation of the *Festuca* allele frequency for each SNP and population are given in table 1.

The mean frequency of the *Festuca* allele in the cv Lueur was found of 0.418, quite close to that of the F1 hybrid population with, in both cases, a genotype distribution highly departing from equilibrium ($X^2_{2\ ddl} = 54.78$, $X^2_{2\ ddl} = 40.45$, resp., $p<0.001$), due to an excess of *LLFF* genotypes. On the other hand, the

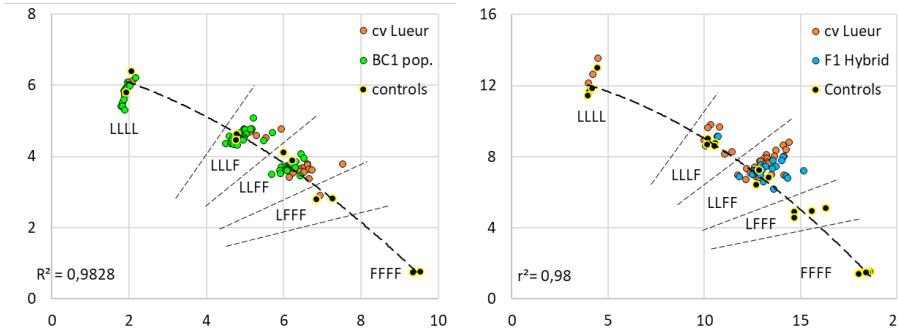


Fig. 1. Scoring into the 5 expected genotype classes at the SNP mapped on position 448 in the scaffold 14459 on the linkage group 1 [2] in 3 tetraploid populations of *L. multiflorum* X *F. glaucescens*: a F1 hybrid, the cv Lueur and a population from one generation of backcross into *L. multiflorum* (BC1). The dotted curve is the quadratic regression between both fluorescent signal across pure DNA of each parent species, *LLLL* and *FFFF*, and the three according mixtures of *LLLF*, *LLFF* and *LFFF* genotype.

BC1 population clearly appeared to be in equilibrium ($X^2_{2\ ddl} = 2.45$, p=0.29) for a frequency of the *Festuca* allele of 0.126, although significantly lower than the value of 0.25 expected from a BC1 population ($X^2_{2\ ddl} = 132.50$, p<0.001). No significant difference of frequency could be found between SNPs within the cv Lueur in contrast to the BC1 population ($X^2_{5\ ddl} = 51.06$, p<0.001). This was mostly due to the SNP mapped on the LG1, in frequency of 0.263 and quite close to the frequency expected in a BC1 ($X^2_{4\ ddl} = 4.23$, p= 0.38).

More results will be needed to support the chromosome mapping of the SNPs and to confirm whether difference of allele frequency across Festulolium populations and/or SNPs can be attributed to selection, either indirectly from fitness or because of breeding. However, these first results are encouraging; they largely confirm what is known about chromosome inheritance in Festulolium derived from *F. glaucescens* [2], they could open to an increasing throughput genotyping in Festulolium, likely to include all *Festuca* sources of Festulolium.

Table 1. *Festuca* allele frequency as estimated from *LLLL* : *LLLF* : *LLFF* genotype distribution at 6 SNPs in 3 Festulolium *L. multiflorum* X *F. glaucescens* populations.

Linkage group	Sequence Position [2]	BC1 LLLL:LLLF:LLFF	freq.	cv Lueur LLLL:LLLF:LLFF	freq.	F1 LLLL:LLLF:LLFF	freq.
LG1	7387_4448	16:24:19	0.263	4:14:42	0.408	0:2:23	0.480
LG2	2066_106870	41:16:2	0.085	1:18:41	0.417	0:2:23	0.480
LG4	5341_49891	32:23:4	0.131	5:19:36	0.379	0:0:25	0.500
LG5	6484_5020	39:16:4	0.102	2:12:46	0.433	0:3:22	0.470
LG6	14459_14639	42:15:2	0.081	1:29:30	0.371	0:0:25	0.500
LG7	336_119185	40:15:4	0.097	0:0:60	0.500	0:0:25	0.500
	All SNPs	210:109:35	0.126	13:92:255	0.418	0:7:143	0.488

References

[1] Byrne, S.L., Nagy, I., Pfeifer, M., Armstead, I., Swain, S., Studer, B., Mayer, K., Campbell, J.D., Czaban, A., Hentrup, S., Panitz, F., Bendixen, C., Hedegaard, J., Caccamo, M., Asp, T. (2015): A synteny-based draft genome sequence of the forage grass Lolium perenne. The Plant Journal 84, 816–826.

[2] Kopecký, D., Loureiro, J., Zwierzykowski, Z., Ghesquiere, M., Dolezel, J. (2006): Genome constitution and evolution in Lolium × Festuca hybrid cultivars (Festulolium). Theor Appl Genet 113:731–742.

[3] Kopecký, D., Bartoš, J., Lukaszewski, A.J., Baird, J.H., Černoch, V., Köllliker, R., Rognli, O.A., Blois, H., Caig, V., Lübberstedt, T., Studer, B., Shaw, P., Doležel, J., Kilian, A. (2009): Development and mapping of DArT markers within the Festuca - Lolium complex. BMC Genomics 10, 473.

[4] Semagn, K., Babu, R., Hearne, S., Olsen, M. (2013): Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): overview of the technology and its application in crop improvement. Molecular Breeding. 33(1), 1–14.

 DOI: 10.5507/vup.21.24459677.34

Detection of genome-specific SNP polymorphism in the *Festuca*/*Lolium* complex

Elza Neau[1], Philippe Barre[1], Sebastian Blugeon[1], David Kopecký[2], Marc Ghesquière[1]

[1] INRAe/URP3F – Lusignan 86600, France
[2] Institute of Experimental Botany, Šlechtitelů 31, CZ-78371 Olomouc, Czech Republic

Marc.Ghesquiere@inrae.fr

Abstract. Festulolium hybrids (*Lolium* sp. X *Festuca* sp.) are a promising avenue for forage breeding. However, the lack of genotyping tools suitable for interspecific hybridization may hinder this prospect. Therefore, we investigate the sequence polymorphism (SNP) among the main ryegrass and fescue species involved in Festulolium hybridization. The aim is to develop KASPar marker genotyping, for higher throughput and more time- and cost-efficiencies than employed during traditional methods. The DNA sequences of *L. multiflorum* and three related *Festuca* sp.: *F. pratensis*, *F. glaucescens* and *F. arundinacea* were aligned onto the reference sequence of *L. perenne*. The sequence polymorphism observed within the *Festuca*/*Lolium* complex closely mirrored the known genome relationships previously observed by cytological investigations. The hexaploid species *F. arundinacea* is confirmed to have evolved following a relatively recent amphiploidization event with a low rate of genome-specific SNPs in contrast to its two ancestor species, *F. pratensis* and *F. glaucescens*. Following the successful primer designs for 705 consensus sequences, 296 SNPs were identified, from which 64 *F. pratensis* genome-specific and 86 *F. glaucescens* genome-specific SNPs as suitable tools for genotyping Festulolium hybrids.

Keywords: SNP markers, KASPar markers, *Festulolium* hybrids, Interspecific hybridization, *Lolium multiflorum*, *Festuca glaucescens, Festuca pratensis*

1 Introduction

Festulolium are interspecific hybrids which for a long time have been used in forage breeding programmes with the aim of combining the complementary advantageous traits found in their parental *Lolium* and *Festuca* species. However, genotyping, which is now used commonly to assist in plant breeding, has to take into account the high linkage disequilibrium in such hybrids in order to determine which molecular markers are the most effective. Genotyping by next generation sequencing using SNPs appears to be the ideal technology to employ

both in respect of number of markers generated and their additive inheritance, but the technique is expensive, time-consuming and may possibly require genome coverage of unnecessary high density. As an alternative for interspecific hybridization, competitive allele-specific PCR (KASP) markers [1] could better meet these requirements. As a precursor study, this paper reports the screening of genome-specific SNPs between the three *Festuca* sp. and the two *Lolium* sp. most involved in Festulolium breeding.

2 Material and Methods

Eleven bulks of genomic DNA were prepared involving two to four DNA bulks of the 4 species: *L. multiflorum*, *F. pratensis*, *F. glaucescens* and *F. arundinacea*. DNA sequencing was performed by Novaseq Illumina™technology after conventional *Pst1* digestion, adaptor ligation and barcoding each individual bulk. Following trimming, and discarding nucleotide position in a frequency less than 10, all sequences were aligned on the *L. perenne* consensus map built by Byrne et al. [1]. Selections for genome-specific SNPs were made by identifying candidates with diallelic polymorphism, a prerequisite in developing KASPar markers from SNPs [2] and then, comparing allelic variants across all four species. For each SNP identified as genome-specific, a consensus sequence of 50 bases upstream and downstream of the target nucleotide was defined using all the sequences from the four species and the reference sequence of *L. perenne* [1]. When a SNP was common between more than two species, consensus sequences were further defined between each single pair of species in order to minimize sequence degenerating and possible inconsistency of primer annealing. All selected consensus sequences were transferred to LGC Genomics Ltd (UK) for primer designing.

3 Results and Discussion

From a total of *circa* 14 million aligned positions, 679,516 positions were shared by all bulks. 11,380 positions were selected on the basis of having a frequency ranging from 80 to 1200 reads and where the most frequently occurring base differed in one species compared to the others. 1,111 positions were further selected as diallelics when their frequency across two species or more, contrasted significantly by >0.99 vs <0.01. A final selection to account for PCR constraints ended with a total of 415 SNPs distant by more than 50 bases within the same scaffold, of which, 295 SNPs were mapped within strict non-redundant scaffolds on the *L. perenne* map [1].

If one considers the number of SNPs between two species as an indicator of genome distance, *L. multiflorum* is confirmed as having the closest genome kinship to *F. pratensis* (40 SNPs), then to *F. glaucescens* (68 SNPs) while both *Festuca* sp. are the most distantly related by 88 SNPs (table 1). A total of 105 SNPs and

Table 1. Distribution of the 295 genome-specific SNPs identified in *Lolium multiflorum* (*Lm*), *Festuca pratensis* (*Fp*), *Festuca glaucescens* (*Fg*) and *Festuca arundinacea* (*Fa*). Genome-specificity defined as diallelic polymorphism between single pairs of species, 199 SNPs, and more than two, 1 vs 2, 1 vs 3 and 2 vs 2, by 75, 17 and 4 SNPs (resp). SNPs between *Festuca* sp only in brown, between *Lolium* sp. vs *Festuca* sp. otherwise.

	Lm	*Fp*	*Fg*	*Fa*	[*Lm* – *Fp*]
Lm	-				-
Fp	40	-			-
Fg	68	88	-		0
Fa	1	2	0	-	0
[Lm – Fp]	-	-	27	0	-
[Lm – Fg]	-	31	-	0	-
[Lm – Fa]	-	1	0	-	-
[Fp – Fg]	15	-	-	0	-
[Fp – Fa]	1	-	0	-	-
[Lm - Fg – Fa]	-	11	-	-	-
[Fp - Fg – Fa]	6	-	-	-	-
[Fg – Fa]	0	3	-	-	1

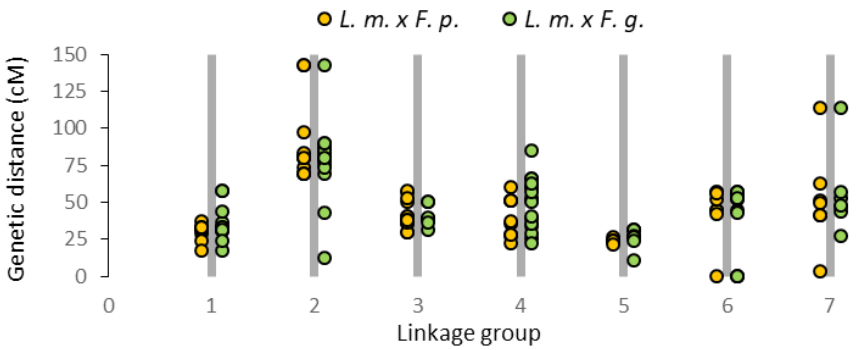


Fig. 1. Mapping onto the *L. perenne* reference map [1] of 64 and 86 primer-designed *Lolium* vs *Festuca*-specific SNPs suitable for genotyping *L. multiflorum* X *F. pratensis* and *L. multiflorum* X *F. glaucescens* Festulolium hybrids (resp). Likewise, the SNPs are distributed across linkage groups by 10-11-14-10-4-8-7 and 14-19-8-17-9-12-7 loci, LG numbering in ascending order.

117 SNPs were found suitable for genotyping *L. multiflorum* X *F. pratensis* and *L. multiflorum* X *F. glaucescens* hybrids (resp). 87 consensus sequence corresponding to 64 SNPs, were successfully primer-designed in the former case, while 115 sequences corresponding to 86 SNPs in the latter. Mapping of those SNPs suggests major centromeric localization, useful for tracking large chromosome events rather than fine recombination. However, the fair distribution of those SNPs across linkage groups joint to simplicity of the KASPar process encour-

ages them to be primarily tested in any Festulolium plant material already well cytogenetically characterized.

References

[1] Byrne, S.L., Nagy, I., Pfeifer, M., Armstead, I., Swain, S., Studer, B., Mayer, K., Campbell, J.D., Czaban, A., Hentrup, S., Panitz, F., Bendixen, C., Hedegaard, J., Caccamo, M., Asp, T. (2015): A synteny-based draft genome sequence of the forage grass *Lolium perenne*. The Plant Journal 84, 816–826.

[2] Semagn, K., Babu, R., Hearne, S., Olsen, M. (2013): Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): overview of the technology and its application in crop improvement. Molecular Breeding. 33(1), 1–14.

 DOI: 10.5507/vup.21.24459677.35

Alterations in leaf lipidome under water deficit and re-watering in *Lolium multiflorum*/*Festuca arundinacea* introgression forms distinct in drought tolerance

Dawid Perlikowski[1,#], Izabela Pawłowicz[1,#], Katarzyna Lechowicz[1], Adam Augustyniak[1,2], Aleksandra Skirycz[3,4] and Arkadiusz Kosmala[1]

[1] Institute of Plant Genetics Polish Academy of Sciences, Strzeszyńska 34, 50-479 Poznań, Poland
[2] Centre for Advanced Technology, Adam Mickiewicz University, 61-614 Poznań, Poland
[3] Department of Molecular Physiology, Max-Planck Institute of Molecular Plant Physiology, Am Mühlenberg 1, 14476 Potsdam-Golm, Germany
[4] Boyce Thompson Institute, Ithaca 14853, New York, USA
[#] equal participation

akos@igr.poznan.pl

Abstract. Mechanisms of drought tolerance have not been fully recognized in forage grasses, including *Lolium-Festuca* species and hybrids. Though, numerous cellular components, including photosynthetic apparatus and antioxidant system, have been proven to be involved in the expression of tolerance, not all the crucial relations existing between these components as well as their cellular functions with respect to drought tolerance, have been clarified. Here, we present the analysis of leaf lipidome profiles under water deficit and subsequent re-watering conditions in two *L. multiflorum/F. arundinacea* introgression forms with contrasting levels of drought tolerance: high drought tolerant and low drought tolerant form. Significant differences in lipidome profiles between the analyzed forms were visible. Furthermore, the leaf lipidome was shown to be more altered by water deficit in the low drought tolerant introgression form. The observed differences could be good indicators of drought survival in forage grasses.

Keywords: drought tolerance, lipidome, *Lolium multiflorum/Festuca arundinacea*

1 Introduction

Lipids are not only the main structural components of cellular membranes but are also involved in energy storage and signal transduction. Because of high susceptibility to peroxidation damage, lipids are affected by environmental stresses, including water deficit [1–2]. The hybrids of *Lolium multiflorum* × *Festuca arun-*

dinacea and their introgression derivatives have been proven to be unique plant materials to dissect mechanisms of drought tolerance in forage grasses [1–3]. Here, we demonstrated that differences in the levels of drought survival observed between the analyzed *L. multiflorum/F. arundinacea* introgression forms were reflected in rearrangements of leaf lipidome under water deficit and subsequent re-watering conditions.

2 Material and Methods

2.1 Plant material

Two tetraploid (4x) *L. multiflorum/F. arundinacea* introgression forms with contrasting levels of drought tolerance: high drought tolerant (HDT) and low drought tolerant (LDT) were used as plant materials. Our earlier study demonstrated that membranes of the HDT introgression form were not damaged under advanced drought (after 10 days of water deficit progression). Contrary, the LDT introgression form revealed significant membrane damage in these conditions. However, after re-watering, the LDT form showed a high potential to regenerate its membranes [2].

2.2 Lipidome profiling

Lipidome profiles were analyzed at five different experimental time points: in the control conditions, on the 3rd (D1), 6th (D2) and 10th (D3) day of water deficit progression, and after subsequent re-watering. The analysis was performed in Max-Planck Institute of Molecular Plant Physiology in Potsdam-Golm, Germany. Briefly, 25 mg of lyophilized leaf tissue was used for lipid extraction in 1 ml of a precooled (15 °C) mixture of methyl-tert-butyl ether (3:1:1, v/v/v) and separated by a reversed-phase C8 column on an Acquity UPLC system (Waters, Milford, Massachusetts, USA). Lipids were further identified using an exactive high resolution mass spectrometer (Thermo Fisher, Waltham, MA, USA) and annotated according to the retention time and the mass-to-charge ratio (m/z) by querying against the in-house database created from 13C isotope-labeled lipids extracted from Arabidopsis. These experimental steps were described in our earlier studies [1–2].

3 Results

Under water deficit and subsequent re-watering, changes in the content of particular lipid fractions occurred in both introgression forms. Significant differences between the analyzed forms were also visible. For example, under advanced

water deficit conditions (D3), pools of monogalactosyldiacylglycerols (MGDG) and sulfoquinovosyldiacylglycerols (SQDG) were reduced, compared the control conditions, in both introgression forms but a pool of digalactosyldiacylglycerols (DGDG) only in the LDT. Pools of phosphatidylethanolamines (PE) and phosphatidylserines (PS) revealed a transient increase under initial water deficit conditions (D1-D2) only in the LDT. Phosphatidylinositols (PI) increased also only in the LDT under advanced drought (D3). Diacylglycerols (DAG) were elevated in the LDT under the whole period of water deficit progression (D1-D3) and triacylglycerols (TAG) at D1 and D3. A content of free fatty acids (FFA) increased in the LDT form only under advanced drought (D3). Unsaturation levels (double bond index, DBI) of phosphatidylcholines (PC), PS and DAG under water deficit increased only in the LDT introgression form.

4 Discussion

The leaf lipidome was more altered by water deficit in the LDT introgression form. In the HDT form, exhibiting a high level of drought tolerance, lipid composition remained relatively unaffected and unsaturation levels of individual lipid classes did not drastically change during a period of water deficit. This phenomenon was correlated with a higher cellular membrane stability and integrity in the HDT form. Differences observed between lipid profiles of two *L. multiflorum*/*F. arundinacea* introgression forms could be good indicators of drought survival in forage grasses. For example, we suggest that a higher amount of FFA, DAG and TAG under stress treatment corresponded to higher membrane damage and lower drought tolerance of the LDT introgression form.

References

[1] Perlikowski, D., Kierszniowska, S., Sawikowska ,A., Krajewski, P., Rapacz, M., Eckhardt Ä., Kosmala A. (2016): Remodeling of leaf cellular glycerolipid composition under drought and re-hydration conditions in grasses from the Lolium-Festuca complex. Front. Plant Sci. 7, 1027. doi: 10.3389/fpls.2016.01027.

[2] Lechowicz, K., Pawłowicz, I., Perlikowski, D., Arasimowicz-Jelonek, M., Blicharz, S., Skirycz, A., Augustyniak, A., Malinowski, R., Rapacz M., Kosmala A. (2020): Adjustment of photosynthetic and antioxidant activities to water deficit is crucial in the drought tolerance of Lolium multiflorum/Festuca arundinacea introgression forms. Int. J. Mol. Sci. 21, 5639. doi: 10.3390/ijms21165639.

[3] Perlikowski, D., Kosmala, A., Rapacz, M., Kościelniak, J., Pawłowicz, I., Zwierzykowski, Z. (2014): Influence of short-term drought conditions and subsequent re-watering on the physiology and proteome of Lolium multiflorum/Festuca arundinacea introgression forms with contrasting levels of tolerance to long-term drought. Plant Biol. 16, 385–394.

DOI: 10.5507/vup.21.24459677.36

High-throughput identification of F1 *Lolium* x *Festuca* hybrids using flow cytometry

David Kopecky[1], Marek Glombik[1,2], Joanna Majka[1,3] and Katerina Pernickova[1,2]

[1] Institute of Experimental Botany, Czech Acad Sci., CZ-78371 Olomouc, Czech Republic
[2] National Centre for Biomolecular Research, Faculty of Science, Masaryk University, CZ-61137 Brno, Czech Republic
[3] Institute of Plant Genetics, Polish Academy of Sciences, PL-60479 Poznan, Poland

kopecky@ueb.cas.cz

Abstract. Identification of F1 interspecific grass hybrids is often a challenge due to the inability to distinguish between the parental morphological features, at least prior to flowering. Molecular and genomic methods have been developed to precisely determine the genome composition of such hybrids. Here, we report on the deployment of flow cytometry (FC) for high-throughput identification of the F1 *Lolium perenne* x *Festuca pratensis* hybrids. The results show that such hybrids can be distinguished from their parents with high confidence, based on the difference in genome size between parents. *L. perenne* used as an internal standard produces the most precise differentiation. Cell suspensions containing *L. perenne* and a putative hybrid always produced two separate peaks on a FC histogram. We conclude that FC can be one of the most powerful and efficient techniques to identify *Lolium* x *Festuca* hybrids with a throughput of about 1000 plants per week.

Keywords: hybrid, Festulolium, flow cytometry

1 Introduction

Interspecific hybridization is a powerful breeding tool to combine the traits from two species into a single organism, or to introgress one or a few traits from one species (usually a wild relative) to another (often elite crop cultivars). In grasses, the combination of high yield and nutrition from ryegrasses and abiotic-stress tolerance from fescues gave rise to numerous Festulolium cultivars [1]. However, identification of the F1 hybrids after interspecific hybridization is unreliable based on the morphology of the plants. On the other hand, molecular and genomic techniques, such as genomic *in situ* hybridization (GISH), Diversity Arrays Technology (DArT) and species-specific SNP platform enable precise characterization of hybrid genome composition [2–4] but are relatively

cumbersome and expensive. Here, we present flow-cytometry as a valuable tool for rapid screening of hundreds of putative hybrid plants.

2 Material and Methods

2.1 Plant material

We used twenty plants of putative F1 hybrids between diploid *L. perenne* cv. Roadstar (2n=2x=14) and *F. pratensis* cv. Skawa (2n=2x=14). The seeds obtained after pollination of emasculated flowers of *L. perenne* by pollen of *F. pratensis* were germinated and once the plants had at least three leaves, they were tested by flow cytometry.

2.2 Flow cytometry and genomic *in situ* hybridization (GISH)

The relative genome size level of each putative F1 hybrid plant was estimated by flow cytometry according to [5]. Nuclear suspensions were prepared from 50 mg leaf tissues of each sample with one parental plant (*L. perenne* or *F. pratensis*). Samples were analyzed using a CyFlow Space flow cytometer (Sysmex Partec GmbH., Görlitz, Germany) equipped with a UV-led diode array. At least 5,000 events were acquired per sample. All 20 plants were further examined by GISH according to [2].

3 Results

At first, we estimated the difference between genome sizes of the parental species, diploids *L. perenne* and *F. pratensis*. Histograms of the relative DNA content contained two well-resolved peaks representing nuclei in G0/G1 phases of the cell cycle, with a very low coefficient of variation (CV) below 1.43%. The difference in position of peaks at the x-axis corresponded to 8.5% difference in the genome size between the two species. Thereafter, we used parental plants together with putative hybrid plant in cell suspensions. When *L. perenne* was used in the suspension, the flow cytometry analysis produced either a single peak, also with low CV (below 1.61%) for two of the 20 tested putative hybrids, or in two separate peaks with very low CV (from 1.09 to 1.77%) for the remaining 18 putative hybrids (Figure 1a). The difference in positions of the peaks corresponded to 5.4% difference (on average) in the genome size between diploid *L. perenne* and putative hybrids. When *F. pratensis* was used in suspensions with putative hybrids, we observed three different results. Eight of the putative hybrids gave two separate peaks (with CV below 1.15%) differing by the average of 3.5% (Figure 1b). Another ten plants gave either a single peak or two peaks so close to each

other that they could not be resolved as two separate peaks by the 'Fit Gauss Peaks' function of CyFlow Space flow cytometer; the CV of this peak was always high (between 2.68 and 3.17%; Figure 1c). The last two plants gave two separate peaks with low CVs (below 1.22%) differing by 7.9 and 8.7%. These were the same two plants which produced single peaks in suspension with *L. perenne*, suggesting their non-hybrid origin.

All 20 putative hybrids were analyzed by GISH. In 18 of these, seven chromosomes showed clear signals of the probe from *F. pratensis*. The other seven chromosomes remained "unpainted", indicating their origin from *L. perenne*, whose sheared DNA was used as the blocking DNA (data not shown). Two plants did not show any signal of the *F. pratensis* probe, suggesting that they were pure *L. perenne* plants that originated by self-pollination, presumably after improper emasculation of the mother plant. These two plants were the same ones that produced single peaks from suspensions with *L. perenne* and with high difference (7.9 and 9.3%) from the *F. pratensis* peak.

4 Discussion

Our results indicate that flow cytometry can be a method of choice for rapid and high-throughput identification of *Festuca* x *Lolium* hybrids. Our suggestion is to use *L. perenne* as an internal standard in cell suspensions of the putative hybrids. However, even *F. pratensis* can serve as a suitable standard: it either produces two separate peaks (one of the hybrid and one of *F. pratensis*) or a single peak composed of the hybrid and *F. pratensis* with a very high CV. Flow cytometry is capable of processing about 200 plants per an 8-hours working day, which is a far greater number compared to conventional GISH (up to 120 plants a week), and far quicker and simpler than any DNA marker tests. Here, we reported only the results for *L. perenne* x *F. pratensis* hybrids, however, the differences between genome sizes of *L. multiflorum* and *F. pratensis* guarantee the same, or even higher resolution [2].

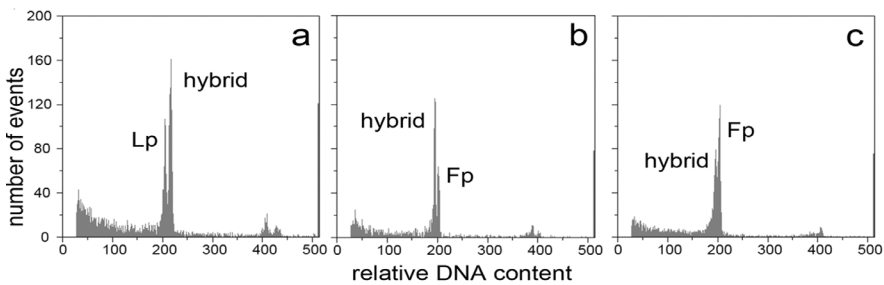


Fig. 1. Flow cytometry histograms of (a) *L. perenne* (Lp) and putative hybrid, and (b,c) *F. pratensis* (Fp) and putative hybrid with separate peaks (b) or a composite peak with high CV (c).

References

[1] Ghesquière, M., Humphreys, M.W., Zwierzykowski, Z. (2010): Festulolium. In: Boller, B., Posselt, U.K., Veronesi, F. (eds.): Handbook of Plant Breeding Vol. 5 Fodder crops and amenity grasses. pp. 293–316. Springer, Dordrecht.

[2] Kopecký, D., Havránková, M., Loureiro, J., Castro, S., Lukaszewski, A.J. et al. (2010): Physical distribution of homoeologous recombination in individual chromosomes of Festuca pratensis in Lolium multiflorum. Cytogenet. Genome Res. 129, 162–172.

[3] Kopecký, D. et al. (2011): Genomic constitution of Festuca × Lolium hybrids revealed by the DArTFest array. Theor Appl Genet. 122, 355–363.

[4] Stoces, S. et al. (2016): Orthology guided transcriptome assembly of Italian ryegrass and meadow fescue for single-nucleotide polymorphism discovery. Plant Genome 9, 1–14.

[5] Dolezel, J., Greilhuber, J., Suda, J. (2007): Estimation of nuclear DNA content in plants using flow cytometry. Nature Protocols 2, 2233–2244.

Exploiting genetic diversity of forages to fulfil their economic and environmental roles

Managing editor Otakar Loutocký
Graphic design, DTP and pre-press Petr Jančík

Published by Palacký University Olomouc, Křížkovského 8, 779 00, Olomouc
Printed by powerprint, s. r. o., Brandejsovo nám. 1219/1, 165 00, Praha-Suchdol

First edition
Olomouc 2021

DOI: 10.5507/vup.21.24459677
ISBN 978-80-244-5967-7 (print)
ISBN 978-80-244-5968-4 (online: iPDF)
ISBN 978-80-244-5969-1 (online: ePub)
VUP 2021/0199, 2021/0200, 2021/0201

This book includes papers presented at the 2021 online meeting of the Fodder Crops and Amenity Grasses Section of EUCARPIA. The theme of the meeting “Exploiting genetic diversity of forages to fulfil their economic and environmental roles“ was presented in four sessions (1) Natural diversity – a valuable source for breeding, (2) Characterizing genetic diversity – the basis for selection, (3) Strategies to optimally exploit genetic diversity and (4) “Minor” and “new” species – solution for future challenges. Parts I to IV of this book correspond to these four sessions. Part V contains the contributions from the Festulolium Working Group Workshop. The book provides a unique source of information on the most recent results on breeding and research of forage species.