



# Use of GBS for Lucerne Variety Distinction

B. Julier<sup>1</sup>✉, P. Barre<sup>1</sup>, P. Lambroni<sup>1</sup>, S. Delaunay<sup>1</sup>, F. Lafaillette<sup>2</sup>,  
M. Thomasset<sup>3</sup>, and V. Gensollen<sup>4</sup>

<sup>1</sup> P3F, INRA, 86600 Lusignan, France  
bernadette.julier@inra.fr

<sup>2</sup> GEVES, Domaine de l'Anjouère, 49370 Erdre-en-Anjou, France

<sup>3</sup> BioGEVES, Domaine du Magneraud, 17700 Surgères, France

<sup>4</sup> GEVES, 711 rue JF Breton, 34090 Montpellier, France

**Abstract.** Distinctness among lucerne varieties, evaluated in DUS tests, is a frequent obstacle to variety registration. Promising varieties may fail the distinction test despite an agronomic plus-value so the genetic progress is not delivered to farmers. The Genotyping By Sequencing (GBS) technology that enables to obtain a large number of markers on pools of individuals was used to evaluate the distinction among 20 lucerne varieties of the European Catalogue. A total of 40 000 polymorphic markers was obtained. All the varieties were statistically different from the others, and the structure was consistent with knowledge on the varieties. Perspectives to use GBS in DUS testing are given.

**Keywords:** *Medicago sativa* · Genetic diversity · Molecular markers  
Genotyping By Sequencing

## 1 Introduction

Despite persistent genetic improvement of lucerne varieties, as showed by VCU tests, variety distinction is often difficult. For this reason, promising varieties may fail the distinction test and be never registered so the genetic progress is not delivered to farmers. Low throughput genotyping methods (AFLP or SSR) gave weak distinctness among lucerne varieties (Crochemore et al. 1996; Flajoulot et al. 2005). The Genotyping By Sequencing (GBS) technology that enables to obtain a large number of markers (Elshire et al. 2011) can be used on pools of individuals (Raineri et al. 2012) and has shown promising results to compare synthetic varieties of perennial ryegrass (Byrne et al. 2013) and lucerne (Annicchiarico et al. 2016). In the present UPOV regulations, molecular markers are not allowed for direct variety distinction, but they are used to structure the genetic diversity for a few species and to choose couples of varieties (applicant varieties and registered varieties) to grow side by side in the field during DUS testing (UPOV 2013). The present study was set up to evaluate the distinction among 20 lucerne varieties chosen in the European Catalogue.

## 2 Material and Methods

### 2.1 Plant Material

Twenty varieties (Table 1) obtained by different breeders have been chosen in the DUS reference catalogue to cover all the range of diversity for autumn dormancy, flower colour, disease (*Colletotrichum trifolii* and *Verticillium alboatrum*) and pest (*Ditylenchus dipsaci*) resistances. All varieties but one (Vernal) are registered in Europe.

### 2.2 Genotyping

Seeds were sown in a greenhouse and leaflets were sampled on four different bulks of 100 individuals for each variety. For each bulk, DNA extraction was carried out with a CTAB protocol. GBS libraries were built by using the restriction enzyme ApeK1 and a specific barcode for each bulk. Libraries were sequenced on an Illumina HiSeq 3000 at the Genomic platform GeT-PlaGe of INRA (INRA Toulouse, France) with a 150

**Table 1.** Description of the 20 alfalfa varieties

Variety	Flower colour <sup>a</sup>				Dormancy <sup>b</sup>	Resistance to <sup>c</sup>			Breeder	Registered in
	C1	C2	C3	C4		Va	Dd	Ct		
Arpège	1	8	2	1	4	7	7	4	Florimond Desprez	2004
Artémis	1	8	2	1	4	6	8	9	Barenbrug	2010
Barned	1	8	2	2	7	5	5	8	Barenbrug	2002
Capri	1	7	2	1	4	7	7	4	Florimond Desprez	1995
Daphné	1	7	2	2	4	5	7	4	Florimond Desprez	1996
Dorine	1	7	3	1	6	3	5	3	Barenbrug	2001
Europe	1	8	2	1	4	5	3	na	Florimond Desprez	1961
Fado	1	8	2	1	4	6	8	6	Florimond Desprez	2012
Félicia	1	7	2	2	4	7	8	7	GIE Grass	2009
Franken Neu	1	5	1	6	4	3	4	na	Schmitz Ernst	1980
Galaxie	1	7	2	2	4	5	7	7	Semunion	2007
Greenmed	1	7	2	2	1	4	7	na	AgriObtentions	2011
Juurlu	3	2	1	1	1	na	na	na	Jogeva PBI	2002
Luzelle	1	6	2	3	3	3	6	na	INRA	1993
Meldor	1	6	2	2	6	4	5	4	INRA	1995
Midi	1	6	2	2	5	4	5	na	Tourneur	2002
Milky Max	1	7	2	2	4	7	8	6	GIE grass	2015
Orca	1	1	8	1	4	2	4	na	Carneau	1966
Verdor	1	7	2	2	6	5	5	3	Barenbrug	2011
Vernal	1	4	4	4	2	na	na	na	Wisconsin and Utah agricultural experiment stations	1953

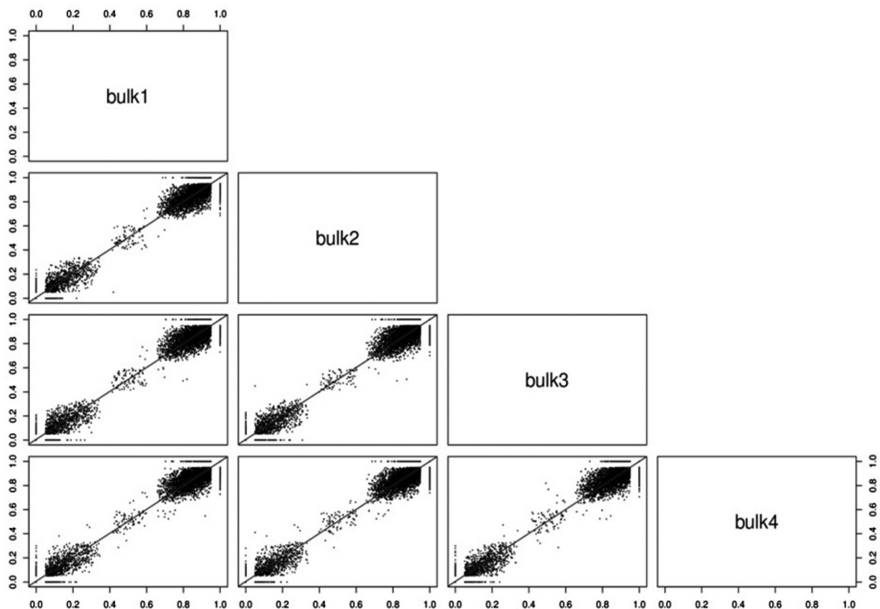
<sup>a</sup> Intensity of flower color expression, from 1: 0–11% of plants to 9: 89–100% of plants with the indicated flower colour: C1: yellow, white or cream, C2: light purple, C3: dark violet, C4: variegated, <sup>b</sup> Autumn dormancy on the US scale ranging from 1 (very dormant) to 11 (non dormant); <sup>c</sup> Va: *Verticillium alboatrum*, Dd: *Ditylenchus dipsaci*, Ct: *Colletotrichum trifolii*; na: non available

base single-end protocol. A Linux pipeline was developed to obtain the allele frequencies. It contains the successive steps: (i) demultiplexing, (ii) trimming of the sequences; (iii) alignment of the sequences on *M. truncatula* reference genome sequence, version 4.0 with bwa, (iv) variant calling based on the GATK programme, (v) calculation of the allelic frequency of each SNP in each bulk obtained by the proportion of the variant allele to the total number of reads, (vi) merging of the files obtained for all bulks into a single file.

Statistical analyses were based on DAPC (Discriminant Analysis of Principal Components) with the procedure *dapc* of *R* software, calculation of  $F_{ST}$  between pairs of varieties and test for significance of  $F_{ST}$  with the procedure *stamppFst* of *R*.

### 3 Results and Discussion

A total of 39 424 markers was obtained. The alleles with a frequency close to 0.25 or 0.75 in the populations were the most numerous. The comparison of the 4 bulks of 100 plants showed a good correlation within each variety (Fig. 1).



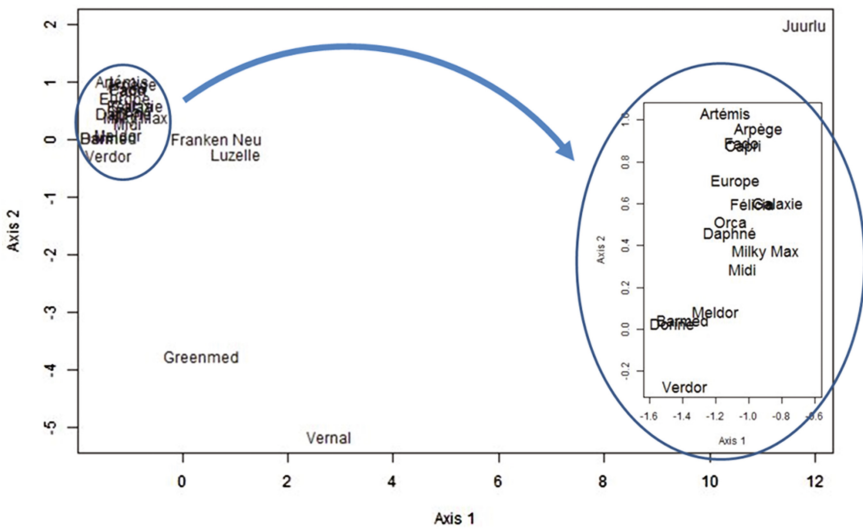
**Fig. 1.** Comparison of allelic frequency in the 4 bulks of variety Galaxie

With the DAPC analysis, five varieties were clearly separated from the 15 other ones (Fig. 2): 1/Juurlu is a variety from Estonia, with a dormancy of 1 and a high proportion of yellow flowers, 2/Vernal has a dormancy of 2, variegated flowers and was bred for Northern USA and Canada, 3/Greenmed is a turf variety selected from

wild *M. sativa* populations, 4/Luzelle is a French variety adapted to grazing that contains one plant of the ssp *falcata* among the 13 parents of the initial polycross, and 5/Franken Neu is a German variety with a high proportion of variegated flowers. The other 15 varieties were all bred in France. When zooming on these 15 varieties, the 5 varieties adapted to the South (Midi, Meldor, Barméd, Dorine and Verdor) had the lowest values on the axis 2 than the 10 varieties adapted to the North (Artémis, Arpège, Fado, Capri, Europe, Félicia, Galaxie, Orca, Daphné, Milky Max) (Fig. 2).

The  $F_{ST}$  calculated between pairs of varieties ranged from 0.075 to 0.207 and were all highly significant ( $P < 0.001$ ). This indicates that all varieties were different from the others.

On the contrary to other studies conducted with a limited number of markers (AFLP or SSR), we obtained a clear distinctness of varieties and a structure that is relevant to previous knowledge on the varieties. The high number of GBS markers obtained in the present study (close to 40 000) probably explained the better discrimination than in a study with 11 Italian landraces but only 2900 markers (Annicchiarico et al. 2016).



**Fig. 2.** Distribution of the 20 lucerne varieties on the first two axes of the DAPC. The small graph on the right is a zoom on the 15 surrounded varieties.

## 4 Conclusion

GBS markers, scored on bulks of individuals, proved efficient to differentiate lucerne varieties. This method could help in DUS testing. The reference collection of lucerne varieties could be genotyped with GBS markers. On this basis, taking example on the use of markers for maize distinction testing, groups of similar varieties could be

established. When a new variety is submitted to registration, the first step could be to genotype it and to assign it to a group. In a second step, the phenotypic distinction tests could be carried out, within the group of similar varieties. Another strategy would be to base all distinction tests on molecular markers and to use phenotypic traits for variety description and VCU evaluation. These results can help the actors of variety registration to propose an evolution of the regulations, for better efficiency of the efforts devoted to lucerne variety distinctness.

**Acknowledgments.** This study was funded the French Ministry of Agriculture (project CAS-DAR Amediluze C2014-05). We thank the Genomic Platform GeT-PlaGe of INRA in Toulouse, France, for sequencing.

## References

- Annicchiarico P, Nazzicari N, Ananta A, Carelli M, Brummer EC (2016) Assessment of cultivar distinctness in alfalfa: a comparison of genotyping-by-sequencing, simple-sequence repeat marker, and morphophysiological observations. *Plant Genome* 9:1–12
- Byrne S, Czaban A, Studer B, Panitz F, Bendixen C, Asp T (2013) Genome wide allele frequency fingerprints (GWAFs) of populations via genotyping by sequencing. *PLoS One* 8: e57438
- Crochemore ML, Huyghe C, Kerlan MC, Durand F, Julier B (1996) Partitioning and distribution of RAPD variation in a set of populations of the *Medicago sativa* complex. *Agronomie* 16:421–432
- Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, Mitchell SE (2011) A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PLoS One* 6:e19379
- Flajoulot S, Ronfort J, Baudouin P, Barre P, Huguet T, Huyghe C, Julier B (2005) Genetic diversity among alfalfa (*Medicago sativa*) cultivars coming from a single breeding program, using SSR markers. *Theor Appl Genet* 111:1420–1429
- Raineri E, Ferretti L, Esteve-Codina A, Nevado B, Heath S, Perez-Enciso M (2012) SNP calling by sequencing pooled samples. *BMC Bioinform.* 13:239
- UPOV (2013) Guidance of the use of biochemical and molecular markers in the examination of distinctness, uniformity and stability (DUS). [http://www.upov.int/edocs/tgpdocs/en/tgp\\_15.pdf](http://www.upov.int/edocs/tgpdocs/en/tgp_15.pdf)