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RESEARCH

Genetic and Environmental Variation in a Commercial Breeding Program of Perennial Ryegrass

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ABSTRACT

Implementation of genomic selection (GS) for the genetic improvement of forage crops, such as perennial ryegrass, requires the establishment of sufficiently large training populations with high-quality phenotype and genotype data. This paper presents estimates of genetic and environmental variance and covariance components, obtained in a training population of 1453 F2 families. These families were produced in 2001, 2003, 2005, and 2007, and they were tested at seven locations throughout Europe. Families were cultivated together with commercial varieties that were used as control. Analyses focused on forage yield (green and dry matter) and six traits scored by visual inspection (i.e., rust resistance, aftermath heading, spring growth, density, winter hardiness, and heading date). Data were analyzed with linear mixed models, including fixed effects (trial and control varieties, within year and location), and random effects (breeding values, pedigree or parents, repeated effects of family or parents within location, and within trial environmental effects, to recover interblock information). Results showed very significant genetic variances for all traits, which provide good opportunities for future GS-based breeding programs. Forage yield showed family heritabilities of up to 0.30 across locations and up to 0.60 within a location. Similar or moderately lower values were found for the other traits. In particular, the heritabilities of rust resistance and aftermath heading were very promising. Genetic correlations between traits were generally low but positive, which increases the potential for multitrait selection.

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Abbreviations: DMY, dry matter yield; GMY, green matter yield; G × E, genotype by environment; GS, genomic selection; PP, parent population; YLT, trial within location and year.

Perennial ryegrass (*Lolium perenne* L.) is an obligate allogamous species with genetic gametophytic self-incompatibility (Cornish et al., 1979). As a result of these properties, *L. perenne* has greater within- than between-family genetic variability. This characteristic has been demonstrated, with proportions that vary depending on the trait, not only in ecotypes (Fernando et al., 1997), but also in selected cultivars (Kölliker et al., 1999), which are heterogeneous populations of individual genotypes.

Perennial ryegrass, as one of the most commonly cultivated species in temperate grasslands, has been subject to substantial breeding activities during the past decades. It In the 1950s and 1960s, breeding companies and public institutes in Europe, Northern America, New Zealand, and Australia performed polycrosses of landraces or ecotypes, which are largely responsible for the modern breeding germoplasm (Conaghan and Casler, 2011). Although initially based on single plant records, selection was later expanded to consider groups of progeny. Since the 1950s, improvements in forage yield have ranged between 1 and 4% per decade (Humphreys, 2005). Several hypotheses have been formulated to explain these relatively low gains (Casler and Brummer,

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2008). These hypotheses include (i) a longer breeding cycle compared with annual grain crops, (ii) an inability to exploit heterosis in commercial cultivars, and (iii) selection based on spaced plants, which show a poor correlation with results in swards (Lazenby and Rogers, 1964; Foster, 1973; Hayward and Vivero, 1984), most likely due to the absence of competing neighbor plants (Elgersma, 1990). In recent years, higher gains have been achieved by taking advantage of the additive genetic variance within half- or full-sib families (Wilkins and Humphreys, 2003; Casler and Brummer, 2008). Considerable selection responses have been obtained in increasing rust resistance and spring growth, and in decreasing aftermath heading (Sampoux et.al, 2011). Further improvements are expected from the application of marker-assisted selection programs, and from the high level of genetic variation among and within cultivars (Wilkins and Humphreys, 2003; Sampoux et al., 2011; Conaghan and Casler, 2011). However, many of the identified quantitative trait loci (QTLs) only explain a small proportion of genetic variance. Moreover, such QTLs are usually determined from a pseudo-F2 mapping population, which make them less transferable to unrelated perennial ryegrass material.

Genomic selection (Meuwissen et al., 2001) allows the estimation of the joint effects of many markers across the entire genome. Genetic selection appears to be more effective than traditional breeding strategies, especially for complex traits with low heritability that are controlled by many genes (Bernardo, 2008). Although it is widely used in animal breeding, there are only a few examples of GS analyses on real data in plants, specifically in maize (Crossa et al., 2010), wheat (Crossa et al., 2010; Heffner et al., 2011), and barley (Heslot et al., 2013). The actual response in selection schemes has been tested mainly through simulations and should be documented under practical circumstances. Prospects for implementing GS in forage breeding were recently reviewed by Hayes et al. (2013).

Despite the large genetic variability, only a few authors have tried to estimate components of variance in perennial ryegrass. These studies utilized very diverse breeding materials (e.g., ecotypes, cultivars, F1 or F2 families, and synthetic varieties) and performed cultivations under varied environmental conditions, which complicates comparisons. Most analyses were performed on only a few genotypes, or on genotypes derived from a limited number of parents, resulting in low estimation accuracy.

The most investigated trait of perennial ryegrass has been forage yield, reported in nearly all articles. For this trait, the narrow-sense heritabilities vary widely and depend on the variety, location, year, and cut (Devey et al., 1989; Charmet and Ravel, 1991; Ravel and Charmet, 1996; Fernando et al., 1997). Strong genotype by environment (G × E) interactions were reported (Devey et al., 1989; Conaghan et al., 2008a), which were generally not a linear function of the environmental parameters (Brennan and Byth, 1979). Another intensively investigated trait is crown rust resistance, with a large variability in estimates of heritability (Ravel and Charmet, 1996; Reheul and Ghesquiere, 1996; Waldron et al., 1998). Other than genetic and climatic factors, this variability may also be due to the interaction with fungal diseases, which are often difficult to distinguish. Estimates of variance can be biased due to the difficulty in phenotyping low levels of infection.

The present paper describes the analysis of historical data of F2 families produced in a standard commercial breeding program. The aim of the study is to estimate the total amount of genetic and environmental variance for important traits in the breeding program, to evaluate the potential for new and more efficient genomic breeding programs.

MATERIALS AND METHODS

Plant Material, Experimental Design, and Traits

Data were derived from 1453 F_2 families, which were part of a commercial breeding program run by DLF-Trifolium, Store Heddinge, Denmark. Briefly, the breeding protocol included (i) pair-crosses between single plants from different parent populations (PPs). The current breeding program has used 76 PPs to date, all chosen from a seed bank. The seed bank contains normal breeding material from DLF-Trifolium and varieties produced by DLF or other breeding companies. Isolation was ensured by growing plants in cabinets attached to a pollen-tight air circulation system. Cross-pollination was ensured by self-incompatibility (Cornish et al., 1979). After pair-crossing, the following steps were performed: (ii) hand-harvesting of F_1 seeds from both parent plants, pooled together to originate a unique family; (iii) propagation of F_1 families in small plots, placed within a rye field to minimize cross-pollination; (iv) harvesting of F_2 seeds; and (v) field-testing of F_2 families.

Phenotypic records consist of historical data from F_2 families, collected over 7 yr (2001, 2003, 2005, and 2007). F_2 families were sown during the summer and tested over two cropping seasons. Each family was generally sown once, at several locations. Selected commercial varieties were sown across different years, to be used as controls. Plants were grown at seven different locations across Europe and cultivated according to different local management schemes, as detailed in Table 1.

In all locations, fields were divided into trials. Every trial consisted of two subtrials, each of them having 12 different plots (sown in one or two replicates). One sixth of the entries were farmed with the control varieties. Plot dimension varied according to location, year, and management. The field scheme was designed primarily to allow mechanization of the cutting procedures. Plots were 1.5 m wide and 6 to 10 m long, consisting of rows (with 10 cm between each row) and columns, divided by 1 m of very short grass, where cut material was discarded. Border trials were isolated by guard plots.

Data on agronomic traits were collected between 2002 and 2009. The final dataset contained information on:

Table 1. Geographic positions, soil- and management conditions for seven experimental locations.

Location	Position	Soil	Management
Bredeløkke	SE Denmark	clay	conservation man. (4–5 cuts/yr)
Didbrook	S England	clay and stony	first year: conservation man. (5 cuts) second year: simulated grazing (7–9 cuts)
Krefeld	W Germany	clay	conservation man. (5–6 cuts)
Les Alleuds	W France	loam	small plots (only scored traits)
Lopuszna	S Poland	loam	sown early and 2–3 cuts
Vleijmen	S Netherlands	sandy	conservation man. (5–6 cuts)
Flevo polder	S Netherlands	loam	small plots (only scored traits)

1. Forage yield, including green matter yield (GMY) and dry matter yield (DMY), expressed in Kg/m^2 and divided into (i) yield during the first year (1), during the second year (2), and sum of both years (s); (ii) yield from the first cut (01), from the later cuts (L), and sum of all cuts (T). Later cuts were not further divided, due to the different management systems among locations, which implies different number of cuts in different periods.
2. Density, defined as tillers per unit surface during the summer. It was determined by visual scoring on a scale from 1 (no tillers) to 9 (full tillering).
3. Spring growth, defined as the growth rate of ryegrass at the beginning of spring. It was determined by visual scoring on a scale from 1 (no growth) to 9 (high growth rate).
4. Aftermath heading, defined as the amount of stems present 3 wk after a later cut. A later cut was used because the amount of reheading after a first cut is strongly influenced by the developmental stage and by the position of the reproductive meristem in relation to the cutting height (Korte and Watkin, 1985). Aftermath heading was determined by visual scoring on a scale from 1 (intense regrowth) to 9 (absence of regrowth; optimal condition).
5. Winter hardiness, representing the combined effect of the density after winter and the damage caused by snow mold and frost during the cold season. Winter hardiness was determined by visual scoring on a scale from 1 (all plants are dead) to 9 (no damage).
6. Rust resistance, measured during the period of maximum infection and usually scored once during the cropping season. Rust resistance was determined by visual scoring on a scale from 1 (plant completely covered by rust) to 9 (no rust attack). In Les Alleuds and Flevo Polder, rust resistance was scored in small plots in the late summer/autumn after establishment in three repeated measurements, with a cut in between to make the scores independent. Due to the different scoring system, the latter measurements were treated as a different trait (*rust resistance 0*).
7. Heading date, defined as the day on which the plants showed one spikelet per tiller. Heading date was expressed in days since 1 May.

Table 2. Number of plots, mean, SD, minimum and maximum values for all traits.

Trait†	No. of plots	Mean	SD	Min. value	Max. value
GM-1-01	5380	3.17	1.15	0.53	7.23
GM-1-L	4333	4.82	1.39	1.77	9.80
GM-1-t	4331	7.94	2.17	3.22	13.77
GM-2-01	5370	1.86	0.99	0.06	7.11
GM-2-L	4553	3.16	1.53	0.07	6.76
GM-2-t	4553	5.10	1.52	0.82	11.17
GM-T	4318	13.06	3.44	4.49	21.43
DM-1-01	4433	0.71	0.22	0.27	1.43
DM-1-L	3427	1.05	0.20	0.52	1.72
DM-1-t	3425	1.75	0.29	1.12	2.68
DM-2-01	4423	0.44	0.23	0.01	1.47
DM-2-L	3606	0.78	0.22	0.31	1.31
DM-2-t	3606	1.24	0.18	0.58	2.29
DM-T	3411	2.99	0.40	1.96	4.35
Density	1290	5.35	1.42	1	9
Spring growth	2705	5.84	1.85	1	9
Stems regrowth	2192	5.22	2.11	1	9
Winter hardiness	1789	4.91	1.29	1	9
Rust resistance 0	10425	5.79	1.96	1	9
Rust resistance	2617	4.94	1.79	1	9
Heading date	2511	26.45	10.71	2	50

† GM = green matter; DM = dry matter; 1 = first year; 2 = second year; 01 = first cut; L = later cuts; t: total in single year; T = total (sum of the 2 yr).

Table 2 shows the number of plots for which data were recorded and the descriptive statistics (mean, standard deviation [SD], minimum, and maximum) for each trait.

Statistical Model

Different single-trait and multi-trait linear mixed models were tested by the *F*-test (for the fixed part) and the Akaike test (for the random part). For most traits, the following model had the best fit to the data:

$$\mathbf{y} = \mathbf{X}_1\mathbf{x} + \mathbf{X}_2\mathbf{k} + \mathbf{Z}_1\mathbf{i} + \mathbf{Z}_2\mathbf{p} + \mathbf{Z}_3\mathbf{i}_1 + \mathbf{Z}_4\mathbf{p}_1 + \mathbf{Z}_5\mathbf{c} + \mathbf{e} \quad [1]$$

where $\mathbf{y}$ is the vector of observations; $\mathbf{X}_i$ are design matrices of fixed factors; $\mathbf{x}$ is the vector of trial effects nested within location and year (YLT); $\mathbf{k}$ is the vector of effects of control varieties, within year and location (analyses run only on controls showed a significant year × location effect; data not shown); $\mathbf{Z}_i$ are design matrices of random factors; $\mathbf{i}$ is a vector of breeding values ~ N(0, $I\sigma^2_i$); $\mathbf{p}$ is a vector of the originating PPs ~ N(0, $I\sigma^2_p$); $\mathbf{i}_1$ is a vector of G × E interactions (accounts for the presence of replicates in certain fields) ~ N(0, $I\sigma^2_{il}$); $\mathbf{p}_1$ is a vector of originating PPs, nested within location ~ N(0, $I\sigma^2_{pl}$); $\mathbf{c}$ is a vector of spatial effects within YLTs ~ N(0, $I\sigma^2_c$); and $\mathbf{e}$ is a vector of random residuals ~ N(0, $I\sigma^2_e$). Z_2 and Z_4 were built by accounting for the presence of two PPs, as explained in Fig. 1.

An additional random factor ($\mathbf{pp}$) was added to check for the presence of an interaction between PPs, which would primarily be due to a dominance effect. In all cases, except for heading date, the pp factor was not significant and could be

	P_1	P_2	P_3	P_4	...	P_i
id_1	0	0	1	1	...	0
id_2	0	1	0	0	...	1
id_3	0	0	1	0	...	1
id_4	1	0	1	0	...	0
...	...	...	...	...	...	...
id_j	0	0	0	1	...	1

Figure 1. Construction of the design matrices Z_2 and Z_4. id_j = F_2 families; P_i = parent populations.

discarded from the analysis (data not shown). Similarly, most reports in the literature have shown that the degree of dominance is low or not easily detectable, due to a strong interaction with the environment (Kearsey et al., 1987; Devey et al., 1989).

Heading date and spring growth were scored in only one location per sowing year. Therefore, the genotype × location interaction could not be estimated for these traits. The following linear models were used for spring growth (Eq. [2]) and heading date (Eq. [3]):

$$\mathbf{y} = \mathbf{X}_1\mathbf{x} + \mathbf{X}_2\mathbf{k} + \mathbf{Z}_1\mathbf{i} + \mathbf{Z}_2\mathbf{p} + \mathbf{Z}_3\mathbf{p}_1 + \mathbf{Z}_4\mathbf{c} + \mathbf{e} \quad [2]$$

$$\mathbf{y} = \mathbf{X}_1\mathbf{x} + \mathbf{X}_2\mathbf{k} + \mathbf{Z}_1\mathbf{i} + \mathbf{Z}_2\mathbf{p} + \mathbf{Z}_3\mathbf{p}_1 + \mathbf{Z}_4\mathbf{c} + \mathbf{Z}_5\mathbf{pp} + \mathbf{e} \quad [3]$$

For *rust resistance 0*, the model included the interaction between all effects and the variable *scoring* (*s*), relative to repeated measurements within year and location.

$$\mathbf{y} = \mathbf{X}_1\mathbf{sx} + \mathbf{X}_2\mathbf{sk} + \mathbf{Z}_1\mathbf{i} + \mathbf{Z}_2\mathbf{p} + \mathbf{Z}_3\mathbf{p}_1 + \mathbf{Z}_4\mathbf{p}_1 + \mathbf{Z}_5\mathbf{c} + \mathbf{Z}_6\mathbf{si}_1 + \mathbf{Z}_7\mathbf{sp}_1 + \mathbf{Z}_8\mathbf{sc} + \mathbf{Z}_9\mathbf{o} + \mathbf{e} \quad [4]$$

where **sx** is the vector of scoring-year-location-trial effects; **sk** is the vector of scoring-year-location-controls effects; $\mathbf{si}_1$ is the vector of breeding values within location and scoring ~ N(0, $I\sigma^2_{\mathbf{sil}}$); $\mathbf{sp}_1$ is the vector of PPs nested within location and scoring ~ N(0, $I\sigma^2_{\mathbf{spl}}$); **sc** is the vector of spatial effects within YLT and scoring ~ N(0, $I\sigma^2_{\mathbf{sc}}$); and **o** is the vector of plots within field, across scoring ~ N(0, $I\sigma^2_{\mathbf{o}}$).

Two different models were run: (i) a single-trait model (one for each trait), to estimate variance components and their standard errors (SEs); (ii) a multi-trait bivariate model that, in addition to the above mentioned parameters, estimates covariances and correlations (with SEs) of each variable between two different traits. The same analyses were run on different subsets of the full data: (i) three subsets containing observations of early-, intermediate-, and late-heading families; (ii) seven subsets containing observations at each location (to test differences in heritabilities among locations).

Variance components were estimated by the restricted maximum likelihood method (REML) with the DMU software package (Jensen et al., 1997; Madsen and Jensen, 2013).

The estimated variance components can be interpreted as follows: σ^2_i is the variance among families tested across locations, determined by additive genetic effects; σ^2_p is the variance among PPs across locations; σ^2_{il} is the G × E variance, assumed to be constant over locations (G × E for single locations were not calculated due to the absence of repeated records in some fields); σ^2_{pl} is the G × E variance due to PPs; σ^2_c is the variance among subtrials within each YLT; σ^2_{pp} is the variance among PPs combinations; σ^2_{sil} is the permanent environmental variance due to repeated scoring (among F2s and within locations and scorings); σ^2_{spl} is the permanent environmental variance due to repeated scoring among PPs, within locations and scoring; σ^2_{sc} is the variance among subtrials within YLT and scorings; σ^2_o is the environmental variance and within plots, across scoring; and σ^2_e is the variance of residuals (i.e., measurement errors and microenvironment effects within plots).

Genetic Parameter Estimates

The estimated variance components were used to derive genetic population parameters, based on the additive biallelic infinitesimal model by Ashraf et al. (2014). Dominance was insignificant (except for *heading date*) and, thus, was ignored. The model was based on the following assumptions: (i) PPs are in Hardy-Weinberg equilibrium; (ii) PPs are unrelated to each other; (iii) parent plants are chosen at random from the PPs; (iv) absence of self-pollination (ensured by self-incompatibility); (v) no intercross among F_1 families; (vi) no selection between F_1 and F_2; (vii) large number of individuals in PPs and the F_1 and F_2 families; (viii) large number of individuals from each parent combination; and (ix) uniform variances across different factors. For the current breeding program, Ashraf et al. (2014) derived the total additive genetic variance within each PP (at any locus) to be:

$$\sigma^2_{A_{P_i}} = 2p_i(1-p_i)a^2 \quad [5]$$

where p is the mean minor allele frequency, *a* is the allele substitution effect (half of the difference among homozygotes), *i* indicates a given locus, and $\sigma^2_{A_P}$ is additive genetic variance within each PP.

F_1s and F_2s are families that have both *among* and *within* genetic variances. For each genotype pool (plants from the same PPs), the additive genetic variance among families was derived to be half of the additive variance in the PPs (Ashraf):

$$\sigma^2_{B_{F2_i}} = p_i(1-p_i)a^2 \quad [6]$$

which, summing all loci, becomes:

$$\sigma^2_{B_{F2}} = \Re^n_{i=1} p_i\left(1-p_i\right)a^2 = \left[\sigma^2_{(1/2)g_{P_1}} + \sigma^2_{(1/2)g_{P_2}}\right] = \left(1/2\right)\sigma^2_{A_P} \quad [7]$$

where *n* is the total number of loci; g_{P_1} and g_{P_2} are the parental genotypes, and $\sigma^2_{B_{F2}}$ indicates additive genetic variance between F_2s was derived to be half of the additive variance in the PPs (the *i* indicates at a given locus). Assuming the absence of selection or genetic drift, this estimate is constant over generations because mating only occurs within families. Variance

among F_2s is the only variance that is directly detectable by observations made on plots. Therefore, the parameters were estimated on the basis of family means.

Four kinds of heritability were defined: heritability within PPs and total heritability across PPs, both location-specific and across locations. Formulas for *heading date* and *rust resistance 0* are not shown because they can be easily computed by adding additional parameters to the denominator. Heritability within PPs across locations (h^2_W) can be calculated by:

$$h^2_W = \sigma^2_i / (\sigma^2_i + \sigma^2_{il} + 2\sigma^2_{pl} + \sigma^2_c + \sigma^2_e) \quad [8]$$

where σ^2_i represents the variance among F_2s. The component σ^2_{pl} must be added to the formula twice because each F_2 originated from two PPs. Heritabilities for each location were not calculated, due to the relatively small amount of data. The location-specific heritability within PPs (h^2_{WL}), which represents a mean value among different locations, can be obtained adding the G × E effects (σ^2_{il}):

$$h^2_{WL} = (\sigma^2_i+\sigma^2_{il})/(\sigma^2_i+\sigma^2_{il}+2\sigma^2_{pl}+\sigma^2_c+\sigma^2_e) \quad [9]$$

Heritability across locations and PPs (h^2_T) can be worked out from Eq. [8], adding $2\sigma^2_p$ to the numerator and denominator:

$$h^2_T = (\sigma^2_i+2\sigma^2_p)/(\sigma^2_i+\sigma^2_{il}+2\sigma^2_{pl}+\sigma^2_c+\sigma^2_e+2\sigma^2_p) \quad [10]$$

Average location-specific heritability (h^2_{TL}) can be computed from Eq. [10], by adding the G × E effects to the numerator:

$$h^2_{TL} = (\sigma^2_i + 2\sigma^2_p + \sigma^2_{il} + 2\sigma^2_{pl}) / (\sigma^2_i + \sigma^2_{il} + 2\sigma^2_{pl} + \sigma^2_c + \sigma^2_e + 2\sigma^2_p) \quad [11]$$

Correlations between traits were computed. Because the effect due to PPs was very small for almost all traits, this paper will only show three kinds of correlations: correlations between breeding values, across locations (Eq. [12]) and within a location (Eq. [13]), and total phenotypic correlation between two traits (Eq. [14]):

$$\rho_{A_1,A_2} = \sigma_{i_1,i_2} / (\sigma^2_{i_1} \times \sigma^2_{i_2})^{1/2} \quad [12]$$

$$\rho_{L_1,L_2} = (\sigma_{i_1,i_2} + \sigma^2_{i_1 1,il_2}) / [(\sigma^2_{i_1} + \sigma^2_{il_1}) \times (\sigma^2_{i_2} + \sigma^2_{il_2})]^{1/2} \quad [13]$$

$$\rho_{P_1,P_2} = (\sigma_{i_1,i_2} + 2\sigma_{p_1,p_2} + \sigma^2_{il_1,il_2} + 2\sigma_{pl_1,pl_2} + \sigma_{c_1,c_2} + \sigma_{e_1,e_2}) / (\sigma^2_{P_1} + \sigma^2_{P_2})^{1/2} \quad [14]$$

where $\sigma^2_{P_i}$ is the phenotypic variance for trait *i*.

Additive genetic variance among single plants within F_2 families ($\sigma^2_{W_{F2}}$) can be theoretically derived. In contrast with the *among families* component, the *within family* component changes every generation due to inbreeding. In F_2s, which consist of offspring of full sibs, the inbreeding coefficient (*F*) takes the value of 1/4. Although a certain degree of dominance may be present, the effect of dominance was ignored because it was neither observable nor estimable as the phenotype data were collected on a family basis. Therefore, $\sigma^2_{W_{F2}}$ can be derived with the following formula (Falconer and Mackay, 1996):

$$\sigma^2_{W_{F2}} = \sigma^2_{A_P} \times (1 - F) = (3/4)\sigma^2_{A_P} \quad [15]$$

and the total additive variance in F_2s is equal to the sum of the among and within components:

$$\sigma^2_{A_{F2}} = \sigma^2_{B_{F2}} + \sigma^2_{W_{F2}} = [(1/2) + (3/4)]\sigma^2_{A_P} = 1.25\sigma^2_{A_P} \quad [16]$$

However, heritabilities for single plants are not estimable, due to lack of knowledge about environmental parameters, such as the number of plants involved in the measurement, competition effects, and the residual variance within each plot.

Table 3. Total phenotypic variance and heritabilities (with SE) for all traits. Heritabilities are expressed as within (w) and across (p) F2 families, across locations (A), and location-specific (L).

Trait†	σ^2_T	h^2_{wA}	h^2_{pA}	h^2_{wL}	h^2_{pL}
GMY-1-01	0.1209	$0.09_{0.015}$	$0.16_{0.028}$	$0.45_{0.021}$	$0.62_{0.021}$
GMY-1-L	0.1373	$0.17_{0.018}$	$0.22_{0.028}$	$0.39_{0.028}$	$0.55_{0.028}$
GMY-1-T	0.2671	$0.15_{0.018}$	$0.18_{0.025}$	$0.45_{0.027}$	$0.57_{0.027}$
GMY-2-01	0.0827	$0.03_{0.013}$	$0.06_{0.021}$	$0.34_{0.024}$	$0.46_{0.025}$
GMY-2-L	0.1012	$0.16_{0.017}$	$0.21_{0.027}$	$0.52_{0.023}$	$0.70_{0.019}$
GMY-2-T	0.2005	$0.14_{0.016}$	$0.16_{0.023}$	$0.48_{0.024}$	$0.60_{0.023}$
GMY-s-T	0.6602	$0.17_{0.017}$	$0.20_{0.026}$	$0.50_{0.025}$	$0.65_{0.022}$
DMY-1-01	0.0060	$0.10_{0.018}$	$0.18_{0.031}$	$0.28_{0.028}$	$0.39_{0.032}$
DMY-1-L	0.0052	$0.17_{0.021}$	$0.23_{0.035}$	$0.34_{0.032}$	$0.48_{0.035}$
DMY-1-T	0.0099	$0.19_{0.022}$	$0.20_{0.026}$	$0.37_{0.034}$	$0.44_{0.035}$
DMY-2-01	0.0042	$0.05_{0.017}$	$0.13_{0.032}$	$0.33_{0.027}$	$0.45_{0.030}$
DMY-2-L	0.0048	$0.22_{0.020}$	$0.28_{0.033}$	$0.45_{0.027}$	$0.61_{0.026}$
DMY-2-T	0.0096	$0.21_{0.021}$	$0.25_{0.029}$	$0.46_{0.027}$	$0.54_{0.028}$
DMY-s-T	0.0248	$0.26_{0.023}$	$0.30_{0.030}$	$0.48_{0.029}$	$0.57_{0.029}$
Aftermath heading	1.5998	$0.26_{0.035}$	$0.34_{0.046}$	$0.50_{0.031}$	$0.59_{0.033}$
Winter hardiness	1.2549	$0.12_{0.034}$	$0.16_{0.049}$	$0.51_{0.046}$	$0.64_{0.042}$
Density	1.5085	$0.12_{0.041}$	$0.17_{0.072}$	$0.37_{0.055}$	$0.59_{0.057}$
Rust resistsance 0	1.9913	$0.21_{0.025}$	$0.26_{0.033}$	$0.28_{0.017}$	$0.37_{0.022}$
Rust resistsance	1.0075	$0.26_{0.033}$	$0.26_{0.039}$	$0.34_{0.039}$	$0.40_{0.040}$
Spring growth	0.6139	–	–	$0.34_{0.031}$	$0.48_{0.031}$
Heading date	27.0528	–	–	$0.53_{0.037}$	$0.67_{0.052}$

† GMY = green matter yield; DMY = dry matter yield; 1 = first year; 2 = second year; s = sum of both years; 01 = first cut; L = later cuts; T = sum of all cuts.

RESULTS

Heitabilities

All traits showed significant genetic variance (Table 3). Green matter yield and DMY displayed very similar patterns (Fig. 2). Location-specific heritabilities were always higher than heritabilities across locations, due to significant G × E effects. Estimates across and within PPs were always significantly different for location-specific heritabilities, but not for heritabilities across locations.

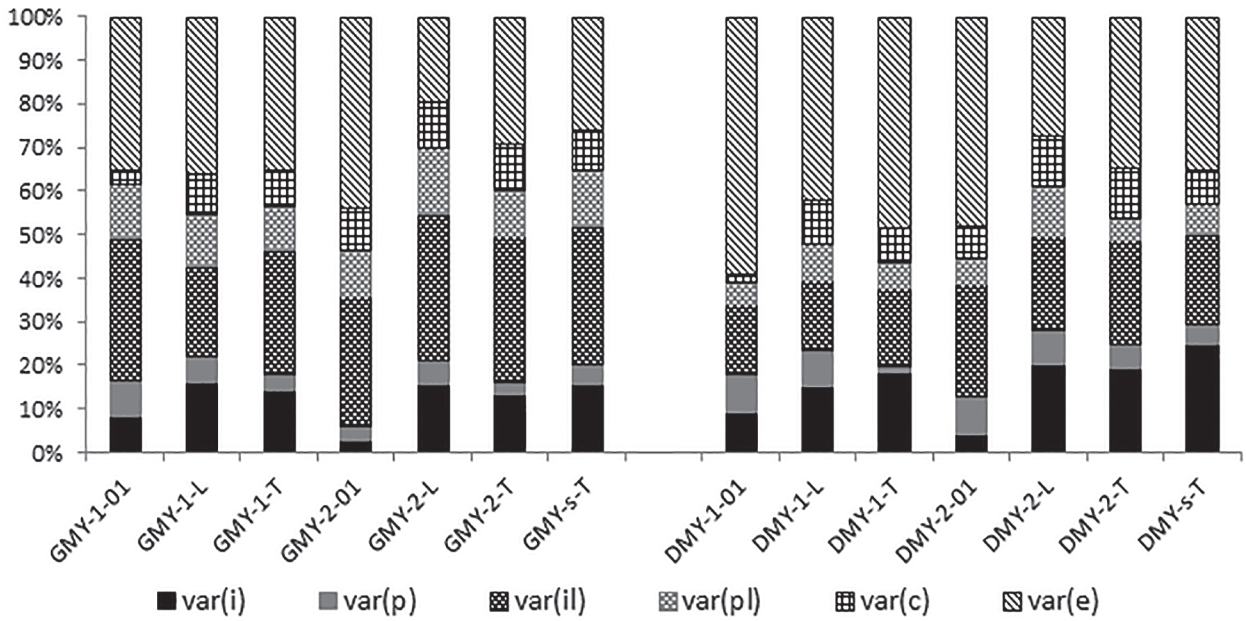

Figure 2. Percentage of variance components over the total phenotypic variance in yield traits. GMY = green matter yield; DMY = dry matter yield; 1 = first year; 2 = second year; s = sum of both years; 01 = first cut; L = later cuts; T = sum of all cuts. var(i) = σ_i^2; var(p) = σ_p^2; var(il) = σ_{il}^2; var(pl) = σ_{pl}^2; var(c) = σ_c^2; var(e) = σ_e^2. Total genetic variance (across parent populations) is given by the sum of σ_i^2 and σ_p^2 (solid fill). See text for variance component definitions.

Comparing GMY-s-T and DMY-s-T, heritabilities across locations were significantly higher for DMY. Values across PPs were 0.30 for DMY-s-T and 0.20 for GMY-s-T. Estimates were not significantly different in the first year and in the first cut of the second year. In later cuts, the proportion of genetic variance in GMY remained at the same level as in the first year. However, the heritabilities for DMY in later cuts were significantly higher compared with those in the first year, reaching values of 0.22 within and 0.28 across PPs (estimates for GMY-2-L were 0.16 and 0.21, respectively). Regarding location-specific heritabilities, GMY always showed higher estimates, mainly due to its higher σ_{il}^2. Across PPs, the difference was always significant; within PPs, the difference was significant only in the first year. Within specific locations, in the second year, the first cut showed significantly smaller heritabilities if compared to later cuts. Across locations, this result was also registered in the first year. A more detailed analysis of the variance components revealed a decrease of the residual variance in the second year for GMY and DMY.

Analyses of the early-, intermediate-, and late-heading family subsets (data not shown) did not reveal significant differences among the subgroups for DMY. For GMY, heritabilities across locations were lower for late-heading and higher for early-heading families, mainly due to differences in G × E interactions and environmental effects within the same field (σ_{il}^2 and σ_{pl}^2). Analyses run within each location gave estimates that were not significant due to their large SEs because of the small number of entries for specific locations.

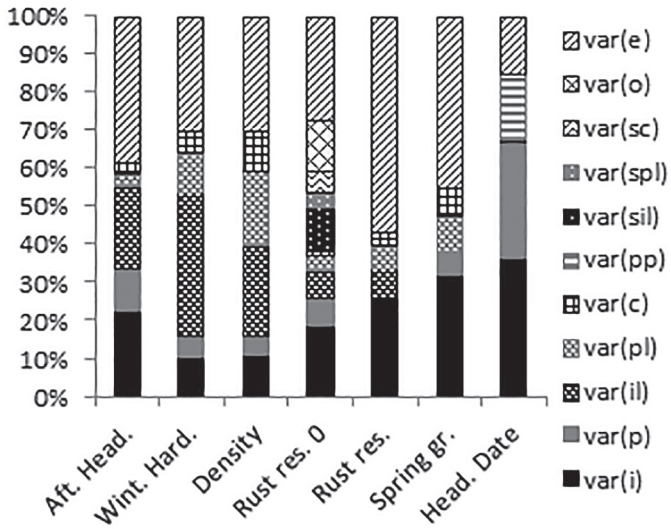

Figure 3. Percentage of variance components over the total phenotypic variance in scored traits. var(i) = σ_i^2 var(p) = σ_p^2; var(il) = σ_{il}^2; var(pl) = σ_{pl}^2; var(c) = σ_c^2; var(pp) = σ_{pp}^2; var(sil) = σ_{sil}^2; var(spl) = σ_{spl}^2; var(sc) = σ_{sc}^2; var(o) = σ_o^2; var(e) = σ_e^2. Total genetic variance (across parent populations) is given by the sum of σ_i^2 and σ_p^2 (solid fill). See text for variance component definitions.

Regarding scored traits (Fig. 3), aftermath heading and rust resistance had the highest estimates across locations, with heritabilities within and across PPs of around 0.26 and 0.34, respectively, for aftermath heading and 0.20 and 0.30, respectively, for rust resistance. Estimates of winter hardiness and density were lower (0.12 within PPs for both traits and 0.16 and 0.17, respectively, across PPs). Location-specific heritabilities, compared with

Table 4. Genetic and phenotypic correlations ($\rho_{P1,P2}$) between different yield traits (with SE). Genetic correlations are expressed as across locations ($\rho_{A1,A2}$) and location-specific ($\rho_{L1,L2}$).

Traits†		$\rho_{A1,A2}$	$\rho_{L1,L2}$	$\rho_{P1,P2}$
Same year, different cuts				
GMY-1-01	GMY-1-L	$0.23_{0.090}$	$0.09_{0.056}$	$0.05_{0.036}$
GMY-2-01	GMY-2-L	$0.82_{0.201}$	$0.25_{0.049}$	$0.07_{0.031}$
DMY-1-01	DMY-1-L	$0.29_{0.115}$	$-0.09_{0.102}$	$-0.07_{0.042}$
DMY-2-01	DMY-2-L	$0.80_{0.173}$	$0.24_{0.065}$	$0.04_{0.038}$
Same cut, green matter and dry matter yield				
GMY-1-01	DMY-1-01	$0.78_{0.061}$	$0.87_{0.076}$	$0.72_{0.038}$
GMY-1-L	DMY-1-L	$0.78_{0.036}$	$0.83_{0.079}$	$0.78_{0.044}$
GMY-1-T	DMY-1-T	$0.70_{0.050}$	$0.73_{0.074}$	$0.70_{0.041}$
GMY-2-01	DMY-2-01	$1.00_{0.076}$	$0.94_{0.081}$	$0.88_{0.040}$
GMY-2-L	DMY-2-L	$0.93_{0.015}$	$0.96_{0.052}$	$0.89_{0.042}$
GMY-2-T	DMY-2-T	$0.95_{0.021}$	$0.97_{0.062}$	$0.86_{0.040}$
GMY-s-T	DMY-s-T	$0.81_{0.030}$	$0.85_{0.058}$	$0.80_{0.042}$
Same cut, different cropping year				
GMY-1-01	GMY-2-01	$0.48_{0.181}$	$0.39_{0.052}$	$0.28_{0.032}$
GMY-1-L	GMY-2-L	$0.79_{0.055}$	$0.43_{0.048}$	$0.39_{0.035}$
GMY-1-T	GMY-2-T	$0.62_{0.070}$	$0.48_{0.050}$	$0.39_{0.034}$
DMY-1-01	DMY-2-01	$0.49_{0.170}$	$0.38_{0.083}$	$0.23_{0.038}$
DMY-1-L	DMY-2-L	$0.76_{0.068}$	$0.35_{0.067}$	$0.34_{0.041}$
DMY-1-T	DMY-2-T	$0.56_{0.075}$	$0.36_{0.070}$	$0.25_{0.037}$

† GMY = green matter yield; DMY = dry matter yield; 1 = first year; 2 = second year; s = sum of both years; 01 = first cut; L = later cuts; T = sum of all cuts.

Table 5. Genetic and phenotypic correlations ($\rho_{P1,P2}$) between scored traits and between yield traits and scored traits (with SE). Genetic correlations are expressed as across locations ($\rho_{A1,A2}$) and location-specific ($\rho_{L1,L2}$).†

Traits‡		$\rho_{A1,A2}$	$\rho_{L1,L2}$	$\rho_{P1,P2}$
Rust resistance 0	rust resistance	$0.84_{0.098}$	$0.59_{0.077}$	$0.17_{0.171}$
Density	winter hardiness	$0.70_{0.198}$	$0.58_{0.112}$	$0.48_{0.074}$
Spring growth	heading date	–	$-0.28_{0.050}$	$-0.24_{0.053}$
Aftermath heading	density	$0.35_{0.155}$	$0.07_{0.129}$	$0.17_{0.076}$
Aftermath heading	rust resistance 0	$0.16_{0.091}$	$0.10_{0.056}$	$0.08_{0.034}$
Aftermath heading	rust resistance	$0.15_{0.101}$	$0.13_{0.103}$	$0.02_{0.048}$
Aftermath heading	heading date	–	$0.23_{0.053}$	$0.31_{0.061}$
Aftermath heading	spring growth	–	$-0.11_{0.061}$	$0.02_{0.070}$
DMY-s-01	heading date	–	$-0.22_{0.046}$	$-0.27_{0.060}$
DMY-s-L	heading date	–	$0.09_{0.043}$	$0.16_{0.056}$
DMY-s-01	aftermath heading	$-0.60_{0.110}$	$-0.35_{0.082}$	$-0.28_{0.050}$
DMY-s-L	aftermath heading	$0.12_{0.090}$	$0.29_{0.084}$	$0.13_{0.057}$
DMY-s-01	winter hardiness	$0.54_{0.152}$	$0.17_{0.114}$	$0.03_{0.061}$
DMY-s-L	winter hardiness	$0.33_{0.119}$	$0.18_{0.089}$	$0.10_{0.057}$
DMY-s-01	spring growth	–	$0.09_{0.049}$	$0.12_{0.050}$
DMY-s-L	spring growth	–	$-0.07_{0.052}$	$0.04_{0.073}$
DMY-s-01	rust resistance 0	$0.01_{0.092}$	$0.00_{0.042}$	$0.01_{0.028}$
DMY-s-L	rust resistance 0	$0.21_{0.066}$	$0.14_{0.044}$	$0.08_{0.027}$
DMY-s-01	density	$0.15_{0.161}$	$-0.16_{0.166}$	$-0.06_{0.082}$
DMY-s-L	density	$0.21_{0.122}$	$0.34_{0.128}$	$0.16_{0.074}$

† Estimates not significantly different than zero are not shown.

‡ GMY = green matter yield; DMY = dry matter yield; s = sum of both years; 01 = first cut; L = later cuts; T = sum of all cuts.

heritabilities across locations, were significantly higher for all traits. This difference was especially large for winter hardiness and density, and was less dramatic for rust resistance. For spring growth and heading date, it was not possible to estimate heritabilities across locations because families were scored only at one location. Estimates for spring growth were 0.34 within and 0.48 across PPs. Heading date showed high heritabilities (0.53 and 0.67 within and across PPs, respectively) that were comparable with the heritabilities for aftermath heading. When analyses were run on the early, intermediate, and late subsets, σ^2_{pp} was not significantly different from zero within the intermediate and early heading groups (data not shown).

Correlations Among Traits

Correlations between the first and later cuts within cropping year are shown in Table 4. Results for GMY and DMY followed the same pattern. First-year estimates showed low (~0) genetic ($\rho_{A1,A2;}$ $\rho_{L1,L2}$) and phenotypic correlations ($\rho_{P1,P2}$). Genetic estimates increased in the second year (up to 0.80), while the phenotypic estimates remained weakly correlated. The correlation between effects of PPs was slightly negative (not shown), affecting the total additive genetic variance and its interaction with the environment. Not surprisingly, GMY and DMY in the same cut always correlated well (Table 4). All estimates (without significant differences between genetic and phenotypic correlations) ranged between 0.70 and 1.00, with higher values in the second year. Due to the algorithm used, an estimate of 1.00 was possible when the correlation was close to the edge of the parameter space. Correlations between the same cuts in two consecutive years (Table 4) were relatively low in the first cut (genetic and phenotypic correlations of 0.49 and 0.28, respectively). In remaining cuts, breeding values correlated well, while the other correlations remained low in the later part of the cropping season (0.30–0.40 for the first cut and 0.35–0.43 for the second cut).

Correlations between scored traits (Table 5) showed *rust resistance* and *rust resistance 0* (only scored in France and the Netherlands) to be highly genetically correlated. Nevertheless, the phenotypic correlation between the two traits was only 0.17. More detailed analyses of different subsets displayed a strong dependency on local climate and scoring system (data not shown). Therefore, these traits could not be treated as a single trait without risking significant drops in heritabilities. Density and winter hardiness were positively correlated both genetically and phenotypically. A negative correlation of about −0.30 was found between spring growth and heading date; thus, families with the highest growth rate in the early spring tended to head earlier. Aftermath heading displayed significant correlations with most of the other traits; it was positively correlated with density and heading date, meaning that less stems were produced in late-heading families. Genetic correlations were found with rust resistance (positive) and spring growth (negative), but in both cases, the variance components were too small to

have any significant effect on phenotypic variance. Genetic correlations were always smaller within a specific location compared with across locations.

Correlations between forage yield and scored traits (Table 5) were calculated from the sum of the two cropping seasons (s) because the traits, depending on the location, were scored on different dates in the two cropping years. Only correlations for DMY are shown because the estimates for GMY and DMY were not significantly different. Dry matter yield showed the highest phenotypic correlations with heading date and aftermath heading. Both traits displayed negative estimates with the first cut; early-heading families and families that produced more stems tended to be most productive in the first part of the farming season. Correlations with later cuts had the opposite sign and tended to be lower, but more stable, across environments. DMY was highly correlated genetically with winter hardiness, especially in the first cut, but did not result in large phenotypic correlations. Slightly positive correlations were found between DMY and spring growth for the first cut, and between DMY and rust resistance in later cuts, for the scorings in Les Alleuds and Moorstraten. The high SEs of parameter estimates for density did not allow estimates for the first cut to be calculated. For later cuts, all values were between approximately 0.15 and 0.35. The G × E effects (remarkable exception) were highly correlated.

Adding the parent effects both across locations and within a specific location did not result in any significant changes, except for the correlation between *aftermath heading* and *heading date*.

DISCUSSION

Forage Yield

The presence of a significant amount of genetic variance across locations will be beneficial for future breeding programs and for the implementation of GS. However, the remarkable difference between location-specific heritabilities and heritabilities across locations indicates the presence of significant interactions between environment and genotypes. The G × E effects on different traits were generally weakly correlated. An interaction was also present between environment and PPs, because the location-specific heritabilities across PPs were significantly higher than those within PPs. This finding confirms the results of other authors (Devey et al., 1989; Wilkins and Humphreys, 2003; Conaghan et al., 2008a) and indicates the need for further investigations into the behavior of these interactions. Such investigations would require at least two replicates per location, together with detailed data about meteorological and soil conditions.

Genotype by environment interactions affected GMY and DMY differently. Across location, DMY always displayed higher heritabilities, showing a generally stronger genetic component. Location-specific estimates were higher for GMY, due to its larger G × E effects. The difference may be explained by day-to-day variations in weather conditions between locations during the cutting period (typically 1–2 wk, depending on the experiment size). For example, samples taken on a rainy day will have significantly higher GMY than samples harvested on days with no precipitation, thereby increasing the environmental variance and its interactions with genotypes at that location.

The highest heritabilities for DMY were found in the last part of the second year. Although this observation may indicate an assurance of the future production of highly persistent varieties, the immediate conclusion of the contrary observation (i.e., that the lowest heritability was found in the first cut) is less encouraging for yield maximization at the most important harvest time-point. This effect may, however, be related to the relatively long cutting period. In spring, when growth rates are exponential and fertilizer levels are not limiting, even a single day of extra growth can add significantly to the observed variation. Because perennial ryegrass varieties are categorized into heading date groups, the first cut will be especially prone to this type of experimental error. A possible solution would be to register and add the day of the cut to the model as a regression covariable within and across the early-, intermediate-, and late-flowering families.

The decrease of residual variance in the second year may be related to a better establishment that makes the cutting conditions more homogeneous, thereby reducing the measurement error. The heritabilities estimated in this paper mostly agree with other publications in sward plots (Frandsen, 1986; Devey et al., 1989; Ravel and Charmet, 1996; Conaghan et al., 2008b). However, the estimates in this paper often appear to be more precise, due to the relatively large amount of data that were included.

As stated in the Results, the first cut and later cuts showed a higher genetic correlation in the second year, probably due to better plot establishment, which stabilizes the yield performance. Phenotypes remained poorly correlated, because of considerable environmental effects and their interactions with genotypes, which represent the biggest components in the models. High correlations between GMY and DMY in the same cut (also shown by Conaghan et al., 2008b), together with the low permanent environmental and residual effects, may allow for indirect selection for DMY using GMY as the selection criterion. This possibility may be especially useful in smaller experiments, in which samples can be harvested under the same weather conditions, or wherever the use of drying equipment would not be cost-effective. The approach would be more problematic for larger experiments because of the variability of the environmental conditions over time.

The low phenotypic correlations between the same cuts in two consecutive years was also shown by Conaghan

et al. (2008b), who reported a small predictability for the yield in the second year based on first-year data. This result could be due to the fact that some families actually produce more in the first year and less in the second year and vice versa. If correct, this explanation would indicate the need to test the breeding material for the whole farming period. Another reason for the result could be changes in weather conditions across years, which directly affect soil conditions, disease incidence, and G × E interactions. This hypothesis, which does not exclude the first one, is supported by the fact that correlations among locations were always lower than genetic correlations, indicating the presence of different permanent environmental effects in the two cropping seasons. On the other hand, the high genetic correlation could allow for a shortening of the trial period, but only in the case of smaller environmental variance. To test these options, it would be worthwhile to test the breeding material in more uniform fields or in locations under similar climatic conditions. However, most breeding programs aim for varieties that perform well across different environments.

Disease Resistance and Agronomic Scores

The relatively small difference between estimates of rust resistance across and within locations indicates a low presence of G × E interactions, at least in locations where this trait was recorded. The model with repeated measurements showed that the variance within families was higher among scorings than among locations. This observation may explain, in part, why *rust resistance* and *rust resistance 0* were not well-correlated. Clearly, more research is needed into the genotype × disease × environment interactions in any stage of the farming season. However, overall, the prospects for creating multi-location resistant varieties seems to be promising.

Other traits showed significant G × E effects. For aftermath heading, this result is in contrast with findings in the literature (Ravel and Charmet, 1996). However, the difference among locations may be due to the scoring system, rather than the environment. Plots were harvested within a range of 1 to 2 wk, which may result in a disparity in phenologic stages between early- and late-heading families. It may be useful to reinvestigate this trait, while ensuring that plants are cut only after a certain level of maturity, or including a regression for the day of harvest (not available for most of the data in the present paper). The high level of G × E effects in winter hardiness is easily explained by the different climatic conditions among the locations, which expose the plants to different environmental stresses. Regarding heading date, results obtained in the subsets showed that σ^2_{PP} may not be due to an additive effect but, more likely, to the consequent use of plants with very similar heading dates for crossing.

Correlations among Traits

Correlations between heading date, spring growth, and DMY and between aftermath heading and density may be due to pleiotropic effects (genes regulating earliness for the first and genes promoting vegetative growth for the latter). Concerning the correlations between forage yield and winter hardiness, density, and rust resistance, the immediate assumption is that the most productive families gave the best yields due to increased environmental robustness. This assumption is further stimulated by the observation that correlation with winter hardiness was especially high in DMY-s-01 (immediately after winter), whereas the correlation with rust resistance only appeared in the late part of the season, when attacks usually occur. Similarly, winter hardiness and density may be correlated because lower density was induced by frost damage in families with low winter hardiness.

The higher stem production in early-heading families may arise because of the simultaneous harvest of families belonging to different heading date groups. However, the same correlation was reported by other authors (Humphreys, 1991; Sampoux et al., 2011), who found relationships between early heading, high stem production, spring growth, and high first cut yield. Like with the G × E interactions, it would be interesting to repeat the investigation, basing the cutting operations on the phenologic stage or including a regression on the day of the harvest. For traits that were genetically but not phenotypically correlated, it may be interesting to test the material under more uniform environmental conditions.

In conclusion, for all traits, but especially DMY, rust resistance, heading date, and aftermath heading, very high or moderately high heritabilities were found. These heritabilities are sufficiently high for further selection and GS implementation in the breeding program. F_2 families will be used as a training population, genotyped (with the *genotype by sequencing* technique) and sown again in Bredeløkke in two replicates. Further studies, to be performed with a more balanced design, may be needed to optimize the experimental design and to analyze G × E interactions and the nature of the correlations between traits.

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