

## RESEARCH ARTICLE

## Genomic prediction of forage nutritive value in perennial ryegrass

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## Abstract

**Background:** Despite its importance to animal production potential, genetic gain for forage nutritive value has been limited in perennial ryegrass (*Lolium perenne* L.) breeding. The objective of this study was to phenotype a training population and develop prediction models to assess the potential of predicting organic matter digestibility (OMD) and neutral detergent fiber (NDF) with genotyping-by-sequencing data.

**Methods:** Near infra-red reflectance spectroscopy calibrations for OMD and NDF were developed and used to phenotype a spaced plant training population of  $n = 1606$ , with matching genotype-by-sequencing data, for developing genomic selection models.  $F_2$  families derived from the training population were also evaluated for OMD and NDF in sward plots and used to empirically validate prediction models.

**Results:** Sufficient genotypic variation exists in breeding populations to improve forage nutritive value, and spectral bands contributing to calibrations were identified. OMD and NDF can be predicted from genomic data with moderate accuracy (predictive ability in the range of 0.51–0.59 and 0.33–0.57, respectively) and models developed on individual plants outperform those developed from family means. Encouragingly, genomic prediction models developed on parental plants can predict OMD in subsequent generations grown as competitive swards.

**Conclusions:** These findings suggest that genetic improvement in forage nutritive value can be accelerated through the application of genomic prediction models.

## KEYWORDS

grass nutritive value, plant breeding, precision agriculture

## INTRODUCTION

Perennial ryegrass (*Lolium perenne* L., PRG) is a grass species commonly used in temperate pastoral systems for the production of forage for livestock. It is known for its fast germination and establishment, and ability to tolerate a range of soil and climatic conditions (Gilliland et al., 2021). However, the nutritive value of forage varies significantly depending on the growing conditions, which can impact the productivity of livestock (Dineen et al., 2021).

Forage nutritive value is important in determining the nutritional value of forages for ruminant animals and

is commonly measured by various parameters, including organic matter digestibility (OMD) and neutral detergent fiber (NDF) (Morgan & Stakelum, 1987; Van Soest et al., 1991). OMD is a measure of the fraction of organic matter in forage that is potentially digestible by the animal. NDF, on the other hand, is a measure of the fiber components of forage that are indigestible by the animal, but partially digestible by the rumen microbes. It includes cellulose, hemicellulose, lignin, and other polysaccharides nondigestible by mammalian enzymes. The NDF content of forage is an important predictor of feed intake, digestion rate, and energy utilization by the animal (Van Soest, 1994). Both traits are

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influenced by factors such as the maturity of the forage, the species, and the management practices used during plant growth (Van Soest, 1994). Other direct estimates of digestibility can be found in the literature as well, such as *in vitro* DM digestibility (DMD) (Wilkins & Lovatt, 2011), or indirect measures, such as water-soluble carbohydrates (WSC). WSC is a trait strongly correlated with DMD, and has been used as a target trait of the Institute of Biological, Environmental, and Rural Sciences (IBERS) breeding program at Aberystwyth (Wales) (Colas et al., 2022; Robins & Alan Lovatt, 2016). Understanding the nutritional value traits of forages is crucial for optimizing animal performance and feed utilization in ruminant production systems.

In a study conducted to assess genetic gains in perennial ryegrass breeding over 40 years, McDonagh et al. (2016) found significant positive trends for dry matter yields, while no evidence of a consistent improvement over time was found for forage digestibility. However, the authors highlighted the existence of large differences between varieties in terms of forage digestibility, indicating potential for future improvement. Moderate genetic gains for nutritive value can be partially attributed to costs and labor-intensive reference laboratory analysis for the traits of interest. Near-infrared reflectance spectroscopy (NIRS) can be used as an indirect method to measure plant constituents related to forage nutritive value (Berauer et al., 2020). Forage materials display distinct absorption bands for C–H, N–H, and O–H in the NIR range of the electromagnetic spectrum, enabling quick and routine quantitative estimation of major components, after calibration development against reference laboratory values (Roberts et al., 2004). Once calibrations for the target constituents are generated, NIRS is much less labor- and cost-intensive than direct chemical measurements.

Selective breeding assisted by the use of genetic markers can be used to improve forage nutritive value and productivity of perennial ryegrass (Conaghan & Casler, 2011). Molecular markers can be used to identify genes and genomic regions associated with desirable traits, such as optimal protein content or digestibility (Hayes et al., 2013). For more complex traits, genomic selection (GS) using an array of genome-wide distributed markers has been identified as a tool to support among- and within-family selection (Hayes et al., 2013). Simulations have clearly demonstrated the potential of this approach to increase genetic gain for traits such as forage yield (Esfandyari et al., 2020; Jahufer et al., 2021). It remains to be established as to how the training data should be acquired (single plant level or population level), in order to blend GS seamlessly with a breeding program, maintain the costs, while retaining high predictive ability (PA) of the models (Hayes et al., 2013). Pembleton et al. (2018) used population-based data on swards to predict seasonal biomass yield and heading date, using data acquired in the normal course of the breeding program, and obtained moderate to high PA for the two traits. Another advantage of using population-based data is that some phenotypic traits, such as biomass production, can be assessed only at the sward level. On the other hand, use of nurseries of spaced

plants (SP) to train genomic prediction models could potentially increase the PA of the models, as larger training populations would be feasible and training data would reflect the individual's genotype and phenotype, as opposed to a family mean.

In this study, we developed NIRS-based predictive models for OMD and NDF that enabled phenotyping of (i) a large population of individual plants evaluated in a SP field trial and (ii) a population of  $F_2$  families derived from parental material sourced from (i) and evaluated in swards. Using the spectra, the relative importance of spectral bands in developing these calibrations for OMD and NDF was investigated. Initially, we tested the ability to predict digestibility of  $F_2$  families using only mid-parent values obtained from NIRS-based evaluations of nutritive value carried out on parental plants in a SP nursery. We then developed genomic prediction models for forage nutritive value (OMD and NDF) in these two perennial ryegrass populations and assessed the ability to use models developed on parental material to predict nutritive value in  $F_2$  families using a small subset of common markers.

## MATERIALS AND METHODS

### Trial design and sampling

#### SP field trial

The PRG plant material originates from field trials conducted at Teagasc Oak Park, Co. Carlow, Ireland (52.8567° N, 6.9127° W). The SP field trial consisted of 30 diploid PRG populations each comprising of 60 genotypes (1800 genotypes in total), described previously in Arojju et al. (2018). Briefly, plants were grown in two replicates in a partially balanced incomplete block design, under two managements: frequent cutting (8 cuts per year) and conservation (4 cuts per year). Each replicate contained an additional 5 control (check) genotypes (Donard, Premium, Spelga, Gilford, and Portstewart), clonally propagated and identical across all blocks. The breakdown of the 30 populations was 10 synthetic cultivars, eight full-sib families, eight half-sib families, and four ecotypes; the full details of these populations are provided in Byrne et al. (2017).

Fertilizer was applied at a rate of 75 kg N ha<sup>-1</sup>, 8 kg P ha<sup>-1</sup>, and 31 kg K ha<sup>-1</sup> in mid-February, with a further 40 kg N ha<sup>-1</sup>, 4 kg P ha<sup>-1</sup>, and 17 kg K ha<sup>-1</sup> applied immediately after all but the final harvest each year. Herbage sampling took place over 3 years (2015–2017). In 2015, spring (April/May) and autumn (September) cuts were collected from the plants under frequent cutting management, and summer cut (July) from the conservation management. Cuts will be referred to as 1st cut (spring cut under frequent cutting), 2nd cut (summer cut under conservation management), and 3rd cut (autumn cut under frequent cutting) to follow the chronology of the cuts throughout the year. Heading date (HD) has been observed as days since April 1st in 2014 and 2015 until the first spike had emerged from three tillers of an individual SP. The HD values have been averaged per individual and

used to calculate the days-to-heading (DtH), expressed as days between the heading date and the cut date.

In 2016 and 2017, only samples from the frequent cutting management were collected, from June and April cuts, respectively. Herbage was cut manually with shears at 70 mm above ground level and all efforts were made to discard stems and seed heads from the final samples. Next, samples were dried at 60°C for 48 h and stored in black nontransparent sacks until milling using a Retsch SM100 cutting mill with a 1.0 mm trapezoid sieve.

## F<sub>2</sub> family trial

An F<sub>2</sub> family population was developed by pair-crossing genotypes selected from the SP field trial described above. The selection of parents was not based on forage quality assessments (unavailable at the time) and avoided selecting parental material from the ecotypes. In 2018, a field trial of 266 of these F<sub>2</sub> families was established. Populations grown in swards ( $n = 266$ ) were assessed in 21 trials of 18 populations (with 2 replicates), under two managements (frequent cutting, conservation management). In each experimental group, four plots belonged to control cultivars. Additional information about the experimental setup can be found in Konkolewska et al. (2023). In 2020, material from swards cut at 40 mm above ground level with a Haldrup harvester was collected (July cut—frequent cutting, August cut—conservation management). Next, samples were dried at 60°C for 48 h and stored in black sacks until milling using a Retsch SM100 cutting mill with a 1.0 mm trapezoid sieve.

## Reference laboratory analysis

OMD was estimated using the in vitro neutral detergent cellulase method of Dowman and Collins (1982), as modified by Morgan et al. (1989). NDF concentration was determined using the Ankom Fiber Analyser (Ankom Technology Corporation; AOAC International, 1995, method 973.18) based on the procedure of Van Soest et al. (1991). Sodium sulfate was used in the NDF procedure and NDF values are expressed including ash. Ash concentration was determined by placing samples in a muffle furnace for 16 h at 550°C (AOAC International, 2000, method 942.05).

## NIRS data collection

Milling to powder was performed with a Retsch Mill, model SM100. The scanning of the library of collected samples with NIRS was completed over the 2021 year. For each measurement, a portion of each milled sample was transferred to a glass cell, gently compacted with a cover, and scanned using a XDS Rapid Content Analyzer (FOSS NIR System DK). Scanning was carried out at 2 nm intervals in the range of 400–2500 nm. Spectral information was collected as absorbance expressed as  $\log(1/R)$ , where  $R$  is the

reflectance, and stored as mean spectra in ISIScan (Infrasoft International). Resulting spectra were scanned for visual outliers, exported, and analyzed with R (R Core Team, 2021) in RStudio (RStudio Team, 2021). The calibration subset was built with  $n = 219$  samples, using iterative selection with the Puchwein algorithm (Shetty et al., 2012). The R script used for the iterative calibration sample selection has been embedded in the RShiny framework (Chang et al., 2024) and is available as an online web application at Konkolewska (2022).

## Phenotype data

Box plots representing the distribution of laboratory reference values for NDF and OMD content of samples included in dry forage nutritive value NIRS calibration collected from SPs and F<sub>2</sub> families trials are presented in Figure S1. Table S1 presents the breakdown of samples collected annually. The summary statistics of the calibration subset are presented in Table S2.

## NIR-based trait prediction

Three linear models have been considered for model training: Partial Least Squares regression (PLS), Elastic net, and K-Nearest Neighbors Localized Weighted PLSR (KNN-LWPLSR, abbreviated to LWPLSR). For PLS, the function *pls* package was used (Mevik et al., 2020), with scaling of data enabled. The Elastic net model was implemented with the *glmnet* package (Friedman et al., 2010), with centering and scaling enabled. LWPLSR was implemented with the *rchemo* package (Lesnoff, 2021). The optimal combination of the model with spectral and mathematical treatments was selected for each trait of interest. Full details of the chemometric modeling can be found in the Appendix and Figures S2 and S3. Optimal calibrations (Table S3) were applied on the whole data set of NIR scans. Models were re-trained on a full calibration subset ( $n = 219$ ) and used for prediction of forage traits on the full data set of NIR spectra ( $n = 16\,080$ ). Trait correlations were calculated with the *corrplot* function (Wei & Simko, 2021).

## BLUES

Estimated marginal means were obtained from linear mixed models with the *lmer* function (Bates et al., 2015). For individual SPs, each plant was analyzed as a fixed effect, model included family, DtH as fixed effects, and random effects due to experimental design (i.e., repetition, block, and block in a repetition). For F<sub>2</sub> families, each family was analyzed as a fixed effect, with management and repetition under management as fixed effects as well. Marginal means and the confidence intervals were estimated with the *emmeans* package (Lenth, 2022). An analysis of variance (ANOVA) was performed using the *aov* function.

## SP family phenotypes to predict F<sub>2</sub> family performance

SP families were classified according to a set criterion: families were marked as being “top” if their BLUEs were higher than the 70th percentile for OMD or falling under the 30th percentile for NDF (Figure S4). Next, among the F<sub>2</sub> families, if a family had two parents originating from the best-SPs families, it was classified as “Top two parents.” Similarly, if only one parent came from the best SP family, the F<sub>2</sub> family was classified as having “One top parent”; if both parents came from the lower-scoring plants, the family was then classified as “Other.”

## Genotype data

### SP

Genotype data were acquired following a protocol described previously by Elshire et al. (2011) and data analyzed by Arojju et al. (2018). Briefly, leaf material was sampled from each SP, freeze-dried, milled, and the genomic DNA was extracted. A genotyping-by-sequencing approach (GBS) was used, using single enzyme digestion with restriction enzyme ApeKI, and the resulting libraries were sequenced on an Illumina HiSeq. 2000. Raw genotyping by sequencing data on SPs was previously submitted to NCBI (BioProject ID: PRJNA352789).

### F<sub>2</sub> families

Approximately 0.8 g of seed was germinated in a glasshouse from each of the 266 F<sub>2</sub> families in small pots, and a lawn was allowed to form (ca. 10 days). Genotype data were acquired following a protocol described previously in Konkolewska et al. (2023). Briefly, leaf material was sampled from each F<sub>2</sub> family, freeze-dried, milled, and the genomic DNA was extracted for each family. A genotyping-by-sequencing approach was used, using a double digestion with restriction enzymes *Pst*I and *Msp*I, and the resulting libraries were sequenced on an Illumina NovoSeq. Raw genotyping by sequencing data on F<sub>2</sub> families was previously submitted to NCBI (BioProject PRJNA884386).

### Data alignment

The sequence data from single plants and F<sub>2</sub> families were aligned to the latest reference genome (Nagy et al., 2022). In the case of individual plant genotype data, adaptors were removed using cutadapt (Martin, 2011) before mapping, and putative variants were identified using bcftools mpileup and call (Danecek et al., 2021; Li, 2011). SNPs were filtered using vcftools (Danecek et al., 2011) with a minimum genotype quality of 20 and a minimum minor allele frequency of 0.025 to yield a final variant database consisting of 116 070 SNPs. More information about the alignment and variant calling in the F<sub>2</sub> families can be found in Konkolewska et al. (2023). In an F<sub>2</sub> family, allele frequencies should segregate according to five segregation ratios, 0, 0.25, 0.5, 0.75, and 1, corresponding to the frequency of the nonreference allele (akin to dosage states in an auto-tetraploid). Given the challenge of accurately determining the frequency of

the variant allele with low-depth genotype-by-sequencing data (Uitdewilligen et al., 2013), we have used the approach outlined in Li et al. (2015) and Annicchiarico et al. (2015) to call genotypes. The genotypes of F<sub>2</sub> families were thus called either homozygous or heterozygous (0/0, 0/1, 1/1), without attempting to further classify into the three potential heterozygous states (simplex, duplex, triplex). The resulting data set was filtered to remove sites with greater than 25% missing data, and the final data set covers 109 438 positions in the genome.

### Feature selection

Marker data sets for SPs and F<sub>2</sub> families were obtained with different restriction enzyme protocols, resulting in only a partial overlap of SNPs ( $n = 5178$ ). This shared pool of markers is hereafter referred to as “common SNPs.” For a comparison of PA with common SNPs, we randomly selected 5178 SNPs out of the whole pool of available markers in F<sub>2</sub>. This subset is referred to hereafter as “random SNPs.”

## Genomic prediction models

First, GBS data were encoded as  $-1, 0, 1$  (where  $-1$  is homozygous for the reference allele,  $0$  is heterozygous, and  $1$  is homozygous for the variant allele). Missing data were imputed with the *A.mat* function (rrBLUP package, EM algorithm, max.missing = 0.5). The following models were assessed: ridge regression Best Linear Unbiased Prediction (rrBLUP, mixed.solve function), genomic BLUP (GBLUP, kin.blup function) from the rrBLUP package (Endelman, 2011), and BayesA, BayesB, and BayesC from the BGLR package (Pérez & de los Campos, 2014). Default options were used, unless stated otherwise. K-fold cross-validation ( $k = 10$ ) was used to evaluate the models. The mean PA (Pearson correlation between observed and predicted trait values on the test data) was recorded on each fold. For a random cross-validation, we used pseudo-randomization for division into separate groups, with a set seed to maintain reproducibility. For the leave-one-family-out cross-validation (LOO), in the SP trial data, an entire family was kept apart for validation purposes and the models were trained on the rest of the data. This process was repeated for all SP families and the PA collected at each fold (corresponding to each family). In terms of the prediction on F<sub>2</sub> families data, each time, one F<sub>2</sub> family was left out for validation and the models trained on the remaining data. This process was repeated for all F<sub>2</sub> families; the PA was calculated based on all predicted and observed values for the F<sub>2</sub> families, across all LOO folds. In terms of prediction of phenotypes of F<sub>2</sub> families, based only on SP training data, a reduced marker data set with SNPs common between SPs and F<sub>2</sub> family data sets (5178 SNPs) was used for model training (rrBLUP or GBLUP), with phenotype data only from SPs. For more information on common SNPs, please refer to Materials and Methods and Supporting Information in Konkolewska et al. (2023). Briefly, SNPs obtained for SPs and F<sub>2</sub> families were derived using different restriction enzymes protocols. The resulting marker data sets overlap in around 5% that constitute the shared common SNPs.

Models were then validated on  $F_2$  family genotype data, and the Pearson correlation between the observed and predicted traits was recorded. Two scenarios were considered: (i) training data included only SPs related to  $F_2$  families ( $n = 420$ ) and (ii) training data included all SPs ( $n = 1606$ ). For comparison, we also assessed the PA within  $F_2$  families, when using randomly selected 5178 SNPs. In order to compare obtained PA results from separate approaches, we analyzed the normality of variances of the mean PA estimates from all iterations and normality of the distribution of the estimates, and then performed an unpaired  $t$ -test ( $H_0$  = the means of the two populations are equal).

## RESULTS

### Phenotyping a large-SP perennial ryegrass population

Substantial phenotypic variation was observed for both forage parameters in both SPs and  $F_2$  families (Figure S1). As expected, we observed a strong negative correlation between OMD and NDF (Figure S5).

Distributions of NIR-predicted forage trait values grouped by season and by year are presented in Figures S6 and S7. Among the SPs, the lowest NDF values for the spring cut were observed in 2015, followed by 2017, and the highest in 2016. Similarly, the highest

OMD values were observed in the spring cuts of 2015 and 2017, with the cut in 2016 characterized by a lower OMD. This could be related to the fact that the sampling period of 2016 was relatively late (in early June) compared to first sampling period in 2015 and 2017 (April/May, April, respectively). With ANOVA, we found that the effect of family was significant and that the variation between season and year group means relative to the variation within the groups was high (with  $F = 15.88$  for OMD,  $F = 18.86$  for NDF, with 34 and 14821 degrees of freedom).

BLUEs were obtained for spring quality for each of the SP families, cultivars, and ecotypes (Figure 1). In terms of nutritive value, ecotypes, and control plants were in general of lower quality, with the highest quality (lowest NDF and highest OMD) observed in half-sib and full-sib families. Overall, we found differences between family means to be significant, with a high variation among the SP families (Table S4).

### Relative importance of spectral bands in OMD and NDF calibrations

Regression coefficients for different wavelengths obtained in optimal predictive models for the prediction of digestibility traits (NDF and OMD) with PLS and Elastic net were visualized using saliency maps as shown in Figure 2.

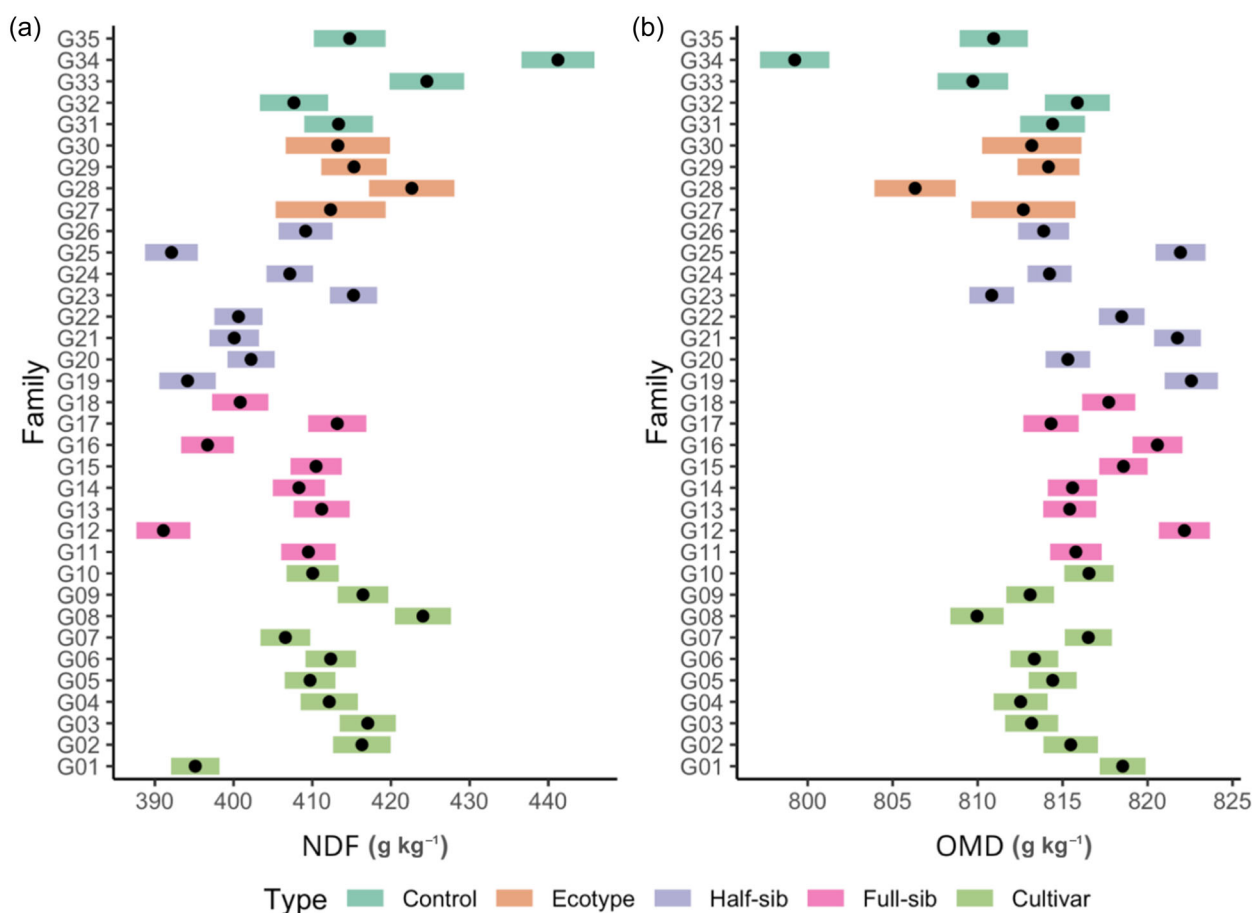


**FIGURE 1** BLUEs with confidence intervals for each of the 35 families, colored according to the origin of the plants. (a) Results for NDF and (b) results for OMD (spring quality).

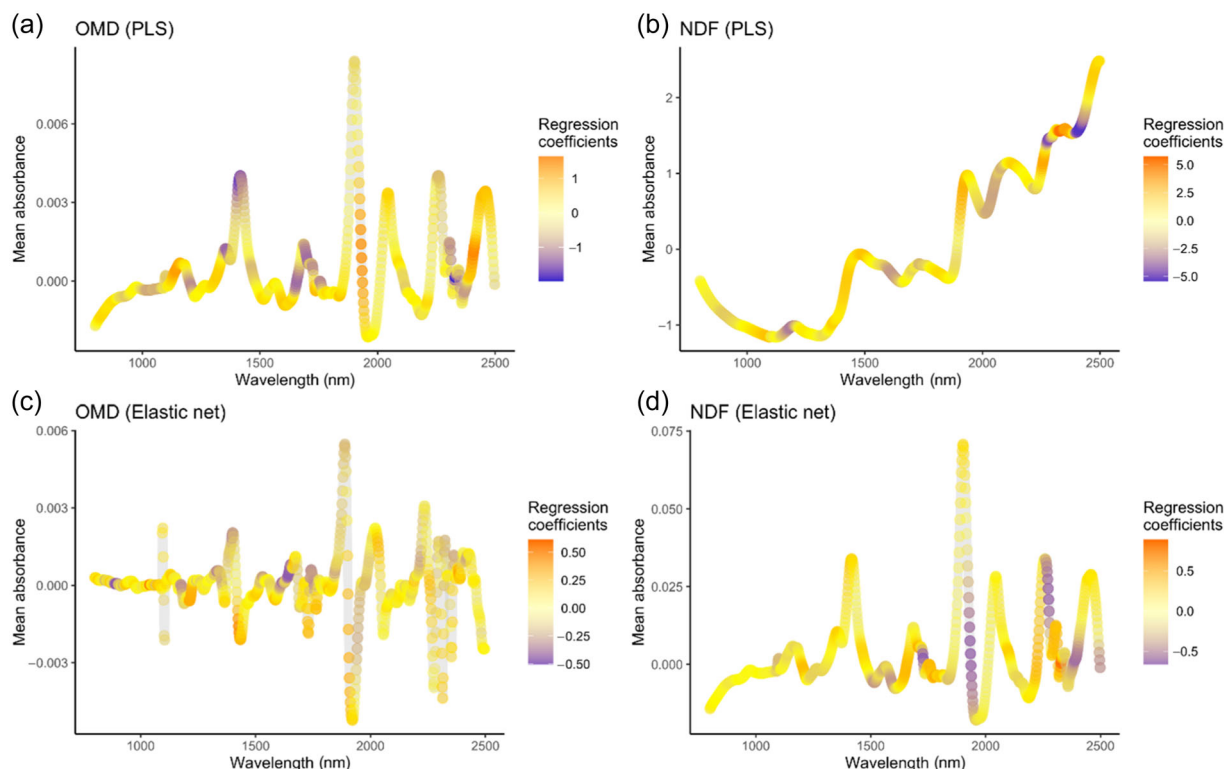


**FIGURE 2** Saliency maps for the best prediction calibrations for organic matter digestibility (OMD) and neutral detergent fiber (NDF). (a, b) Models built with PLS and (c, d) models built with Elastic net. Mean spectra absorbance, with the corresponding mathematical treatment applied, are drawn with gray circles. Non-zero regression coefficients from the models are plotted according to the color scale: negative in blue and positive in red, with the mid-point set at 0 in yellow.

Calibration for OMD comprised 81 wavelengths, covering multiple regions of the NIR spectra, relating to the basics of the measurements and vibrational frequencies of the molecular bonds. Important regions include the C–H 1st overtone, the C–H stretch 1st overtone, the O–H combination band, and the C–H stretch/C–H deformation.

Calibration for NDF comprised 39 wavelengths. Interestingly, two wavelengths already described as strongly negatively correlated with OMD ( $\lambda = 1712, 2328$  nm) were strongly positive predictors of NDF. We found that samples with higher NDF (and, conversely, lower OMD) tended to have higher absorbance in the vicinity of the two wavelengths.

### Phenotypes on parental plants can predict forage nutritive value in subsequent generations grown as competitive swards

In addition to the SPs described above, a trial of  $F_2$  families, grown as competitive swards, was evaluated for forage nutritive value. The obtained ANOVA results indicated statistical differences between the  $F_2$  families (Table S3).

In order to evaluate how well parental midpoint phenotypes can predict performance in  $F_2$  families, we tested two scenarios: (i) using individual SPs phenotypes and (ii) using family means of SPs. Overall, higher PA was achieved when using individual SPs phenotypes (Figure 3).

Obtained BLUEs for  $F_2$  families for traits of interest were also visualized with confidence intervals (at 95%) in Figure 4 and related to forage nutritive value evaluations of parental plants. In terms of NDF, out of 266  $F_2$  families, almost 10% ( $n = 26$ ) had two parents from the top SP families (Figure S4) and 44% ( $n = 117$ ) had at least one parent from the top SP families. We found 27  $F_2$  families with BLUE for NDF lower than the 10th percentile, with 4 (15%)  $F_2$  families having two “top” parents. For OMD, 18% ( $n = 48$ ) had two parents from the top SP families and 32% ( $n = 85$ ) had at least one parent from the best SP families. We found 27  $F_2$  families with BLUE for OMD higher than the 90th percentile, with 12 (25%)  $F_2$  families having two “top” parents. Nine  $F_2$  families were classified as having both the highest OMD levels and the lowest NDF levels.

### Genome-wide markers can predict OMD and NDF with moderate accuracy

In a first analysis, the genome-wide markers were used to develop genomic prediction equations for OMD and NDF in the large SP population consisting of 1606 plants with matching genotype and phenotype data. The mean PA for NDF, assessed with rrBLUP and all SNPs (116 070), was at a level of 0.57 for spring NDF, 0.55 for autumn NDF, and 0.43 for 2nd cut silage NDF (Figure 5). Similarly, for OMD, the mean PA was at a level of 0.59 for spring OMD, 0.52 for autumn OMD,

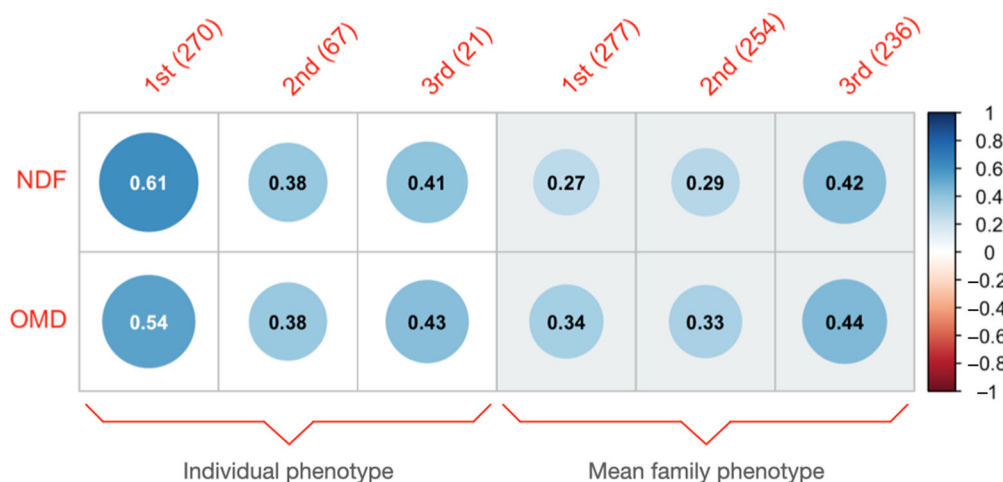


**FIGURE 3** Prediction of forage nutritive value in  $F_2$  families using the parental midpoint: with individual spaced plant phenotypes (white background) or mean SP family phenotypes (gray background). Results are shown as Pearson correlation between the BLUEs of OMD or NDF traits of each  $F_2$  families and the calculated parental mean of the respective digestibility trait from the first, second, or third cut (under frequent cutting management). Numbers in brackets indicate the number of complete pairs used in the calculations.

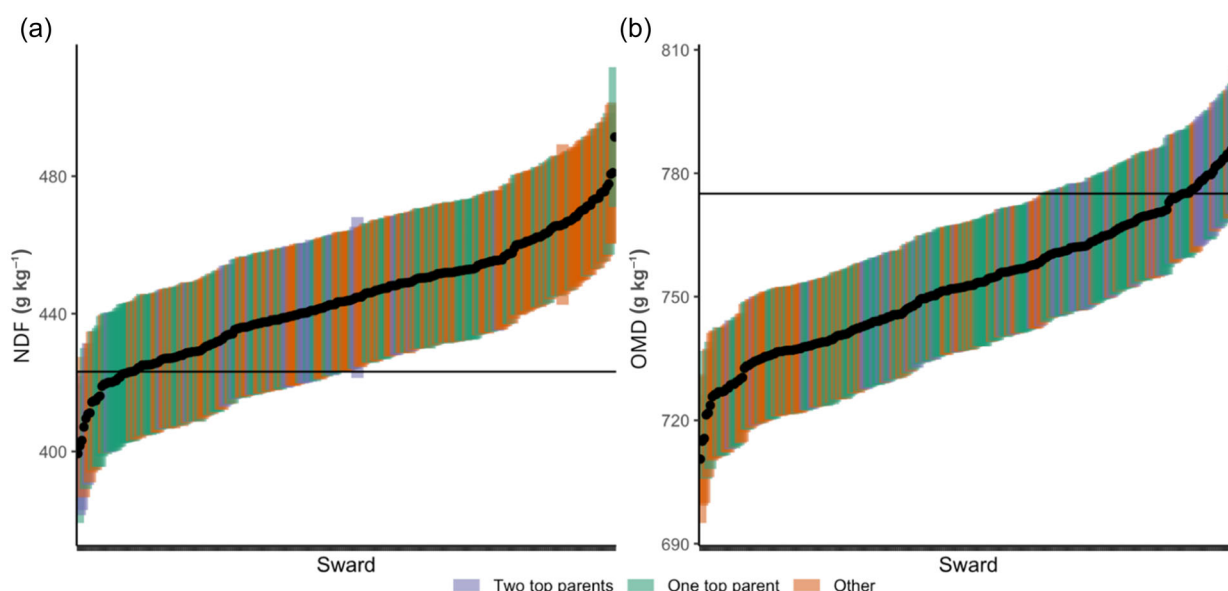


**FIGURE 4** BLUEs with confidence intervals (95%) for each of the  $F_2$  266 swards, colored according to a decision criterion: “Two top parents” (blue), meaning a sward was obtained from spaced plants from families falling under the 30th percentile for NDF, or higher than the 70th percentile for OMD (Figure S10). Similarly, “One top parent” (green) signifies that only one of the spaced plants used for crossing came from the best families. “Other” (orange) means that plants used for crosses were not from the best families. (a) Results for NDF; (b) Results for OMD. A line was drawn at the 10th (NDF) or 90th percentile (OMD). It is worth noting that the feather plots displayed an unusual number of green- and blue-colored bands, which is not typically observed (corresponding to one or two top SP parents). This discrepancy can be attributed to the fact that the selection of parental plants did not include control and ecotype families, which are known for their lower digestibility, whereas these were taken into account when classifying the SP families as top parents.

and 0.56 for 2nd cut silage OMD (Figure 6). Overall, PA obtained for OMD was higher than that for NDF.

In a more challenging LOFO cross-validation, reducing familial relationships in the predictions, we observed a decrease in average PA, with greater spread in PA across folds (Figures 5 and 6). If a family without closely related material in the remainder of the training population was used as the test family in LOFO, the PA dropped considerably.

To evaluate the impact of the prediction model on PA, we evaluated a range of models (rrBLUP, BayesA, BayesB, or BayesC) with both traits. The results indicated that the choice of the genomic prediction

model had no significant effect on the prediction accuracy for these traits. The highest effect on PA was due to trait, as well as the chosen cross-validation strategy. A summary of our results is shown in Table 1.

### Genomic prediction models developed on individual plants outperform models developed with family means

In the case of the SPs, genotypes and phenotypes were collected on individual plants, whereas in the case of the

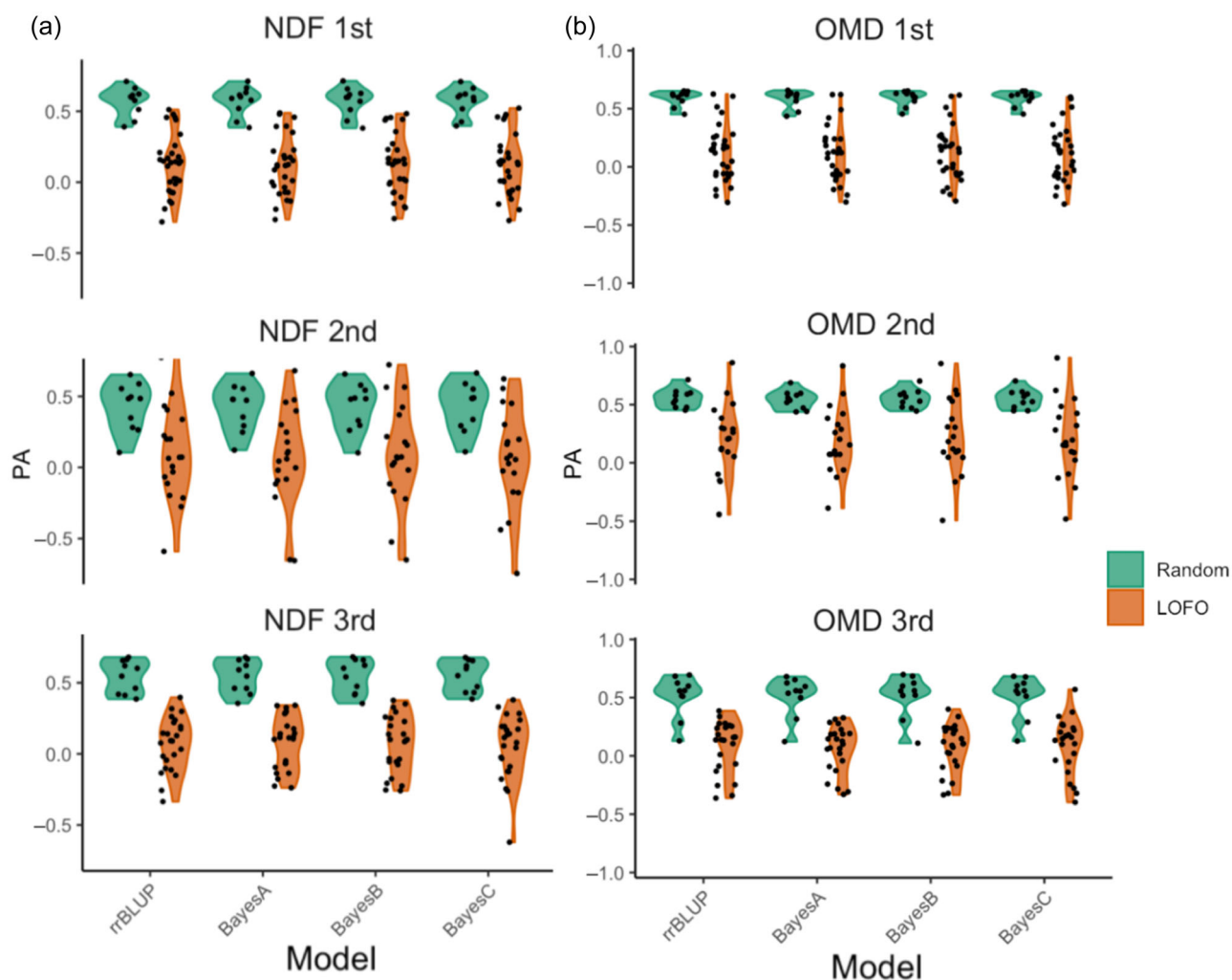


**FIGURE 5** Genomic prediction of forage (a) NDF (neutral detergent fiber) and (b) OMD (organic matter digestibility), 1st cut, 2nd cut, and 3rd cut, in spaced plants, under two different cross-validation protocols: random selection with 10 k-folds or leave-one-family-out (LOFO). LOFO results are shown for families with at least five individuals. PA, predictive ability.

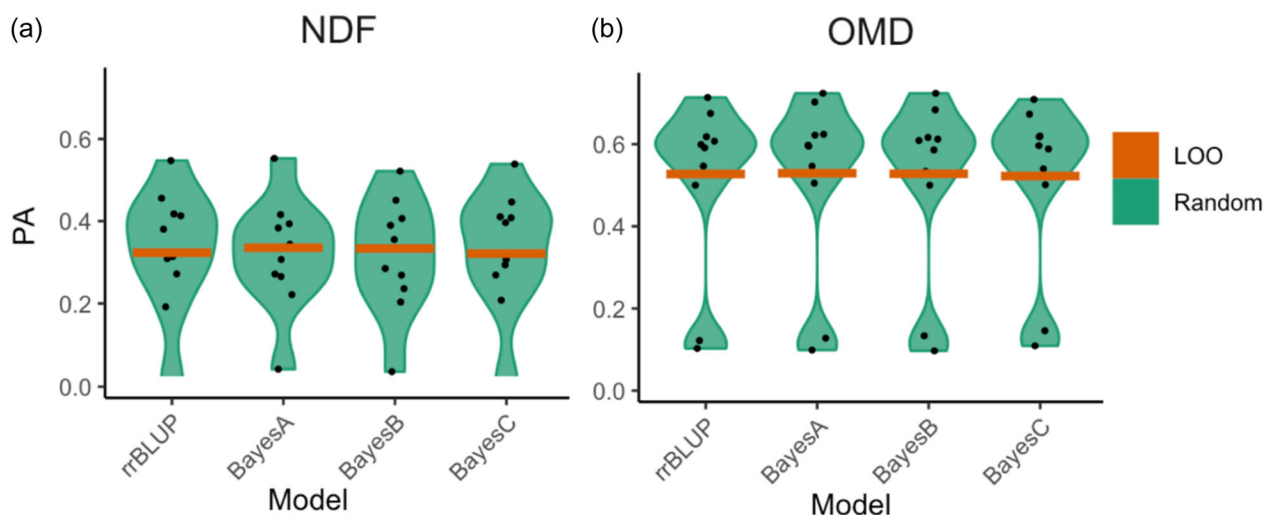


**FIGURE 6** Genomic prediction of forage nutritive value in  $F_2$  families, under two different cross-validation protocols: random selection with 10 k-folds or leave-one-out (LOO). For the random cross-validation, PA (predictive ability) results for all folds are shown. In terms of LOO, PA was calculated as a Pearson correlation between observed and predicted values for each  $F_2$  family.

$F_2$  families, both genotypes and phenotypes were collected from a competitive sward as family means.

In the case of  $F_2$  families, the mean PA for NDF, assessed with rrBLUP, was at a level of 0.33 under

10 k-folds cross-validation; comparable results were obtained under leave-one-out (LOO) testing (PA = 0.32). For OMD, the mean PA assessed with rrBLUP was at a level of 0.51 under 10 k-folds cross-validation and

**TABLE 1** Genomic prediction of quality with rrBLUP models. Results are shown as mean predictive ability.

| Training data   |           |                    | Test data      |       | Trait  |      |                     |      |                     |
|-----------------|-----------|--------------------|----------------|-------|--------|------|---------------------|------|---------------------|
| Genotype (SNPs) | Phenotype |                    | Phenotype      |       | CV     | NDF  | NDF 1st / 2nd / 3rd | OMD  | OMD 1st / 2nd / 3rd |
| SP              | All       | Indiv              | SP             | Indiv | K-fold |      | 0.57 / 0.43 / 0.55  |      | 0.59 / 0.56 / 0.52  |
| SP              | All       | Indiv              | SP             | Indiv | LOFO   |      | 0.13 / 0.01 / 0.16  |      | 0.11 / 0.11 / 0.17  |
| F <sub>2</sub>  | All       | Mean               | F <sub>2</sub> | Mean  | K-fold | 0.33 |                     | 0.51 |                     |
| F <sub>2</sub>  | Random    | Mean               | F <sub>2</sub> | Mean  | K-fold | 0.36 |                     | 0.51 |                     |
| All SPs         | Common    | Indiv <sup>a</sup> | F <sub>2</sub> | Mean  | LOO    | 0.27 |                     | 0.41 |                     |
| Related SPs     | Common    | Indiv <sup>a</sup> | F <sub>2</sub> | Mean  | LOO    | 0.26 |                     | 0.38 |                     |

Note: All SPs,  $n = 1606$ . SPs related to F<sub>2</sub> families,  $n = 420$ . Indiv., Individual phenotype values. Mean, mean phenotype family values (observed in case of F<sub>2</sub> families). All SNPs:  $n = 116\,070$  (SPs),  $n = 108\,355$  (F<sub>2</sub> families); random SNPs:  $n = 5178$ ; common SNPs:  $n = 5178$ . K-fold: 10 k-fold cross-validation.

Abbreviations: CV, cross-validation; LOFO, leave-one-family-out cross-validation; LOO, leave-one-out cross-validation; SP, spaced plants.

<sup>a</sup>Individual phenotype values of SPs for NDF 1st cut.

0.52 under LOO (Figure 6). Similar to genomic prediction in SP, the choice of the genomic prediction model (rrBLUP, BayesA, BayesB, or BayesC) had no impact on PA.

For NDF, PAs among F<sub>2</sub> families were lower than those among SPs, with a significant decrease in PA for NDF: from a mean PA of 0.57 for spring NDF, 0.55 for autumn NDF, and 0.43 for 2nd cut silage NDF when tested in SPs to a mean PA of 0.33 in F<sub>2</sub> families.

However, the predictive abilities for OMD were similar (0.56 for 2nd silage among SPs and 0.51 among F<sub>2</sub> families).

### OMD and NDF in competitive swards can be predicted using genomic prediction models developed on parental plants

We tested two strategies in predicting F<sub>2</sub> family phenotypes using models trained with parental SP genotypes and phenotypes (using 5178 common SNPs): (i) with all available SP data ( $n = 1606$ ) and (ii) using data only from SPs that were used as parental plants to produce F<sub>2</sub> families ( $n = 420$ ). We found no difference whether the model used was rrBLUP or GBLUP (Figures S8 and S9).

Models trained on SPs were used to predict phenotypic values in F<sub>2</sub> families using the common SNPs. A lower mean PA was obtained than when predicting among SPs or F<sub>2</sub> families.

We found that use of wider training data, spanning also SP individuals not directly related to the F<sub>2</sub> families, allowed to obtain a small increase in PAs (Table 1).

For comparison with the empirical assessment across SPs and F<sub>2</sub> families, when predicting within F<sub>2</sub> families with common SNPs, the mean PA was equal to 0.32 for NDF and 0.49 for OMD, whereas using the same number of randomly selected SNPs ( $n = 5178$ ), the mean PA under 10 k-fold cross-validation was equal 0.36 for NDF and 0.5 for OMD.

## DISCUSSION

The rate of genetic gain for forage nutritive value in perennial ryegrass breeding has been modest (McDonagh et al., 2016), and programs require new tools to rapidly

screen plant material to assess the breeding value of selection candidates. In this study, (i) a large population of SPs and F<sub>2</sub> families was phenotyped for OMD and NDF, spectral bands linked with NDF and OMD were identified and (ii) genomic prediction equations with moderate PA were developed for OMD and NDF.

### Sufficient genotypic variation for forage nutritive value exists in breeding material to enable further genetic gain

In this study, we evaluated a large-SP population consisting of 60 plants from each of 30 families, cultivars, and ecotypes. We observed a high phenotypic variation for the traits of interest among the SP families.

In general, plant digestibility was reduced when the harvest date was closer to or after the heading date. This is in agreement with the results obtained by Redfearn et al. (2002), who found that the nutritive value of perennial ryegrass cultivars declined rapidly following seedhead emergence. Over the course of a year, we observed a gradual decline in OMD and a gradual increase in NDF across the three cuts. The lateness of the cut would affect the developmental stage of the plants at the time of the cut, with the weather before the cut having an impact. The authors of Groot et al. (2003) concluded that the temperature increase accelerates both the tiller development rate and the decline rate of cell wall digestibility during aging. Other researchers also observed a decline in in vitro true digestibility and in vitro cell wall digestibility with increased temperatures (Thorvaldsson et al., 2007).

In F<sub>2</sub> families, we observed a lower variance in terms of OMD and NDF under two managements than in the case of SPs (Figure S9). This might relate to the fact that for F<sub>2</sub> families, we are using family means obtained on swards, whereas for SPs, we deal with a high number of individual plants (up to 60 per family). Additionally, sampling on SPs was performed manually at 7 cm and plants were hand-sorted, whereas on F<sub>2</sub> families, sampling was performed with a machine at a lower cut level (4 cm). The observed variance was the highest for OMD under the frequent cutting management.

In general, we observed that swards under conservation management were characterized by lower digestibility

than swards under frequent cutting, which was also shown by other authors Jafari et al. (2003). The variance observed among  $F_2$  families in terms of forage nutritive value is encouraging for further attempts to improve forage nutritive value.

### The relative importance of spectral bands to predicting OMD and NDF

Although laboratory NIRS is a well-developed approach for measuring forage nutritive value parameters, it is labor-intensive because of the substantial pre-processing required to dry, mill, and scan samples in the lab. There are opportunities to use in-field NIRS sensors to capture data on SPs or in competitive swards using harvester-mounted NIRS. In particular, inexpensive sensors targeting limited regions of the spectra may provide opportunities for routine on-farm measurements of forage nutritive value and expand the tools available to farmers for enhanced grassland management. As part of this study, we were interested in identifying the spectral regions that contributed to the predictability of the OMD and NDF calibrations.

In the case of OMD, the calibrations comprised 81 wavelengths covering various regions of the spectra. Regions that positively contributed to OMD have been previously found to be related to the presence of starches, sugars ( $\lambda = 2280\text{--}2330\text{ nm}$ ), and cellulose ( $\lambda = 2340\text{--}2342\text{ nm}$ ). Another region ( $\lambda = 1680\text{--}1684\text{ nm}$ ) could be related to the presence of Ar-CH bonds in proteins (Workman, 2000). The strongest positive association between absorbance and OMD was observed at 1736 nm. In a study by Danieli et al. (2004), researchers found that this wavelength is important for the prediction of NDF. However, no information was provided on whether the wavelength contributed negatively or positively to the NDF prediction. Based on our study, we hypothesize that this signature belongs to more digestible fractions of the structural components of the cell wall. This could potentially relate to the presence of pectins (Vetharaniam et al., 2014) or hemicellulose, such as arabinoxylans (Colas et al., 2022). Alternatively, the related NIR region also covers the -SH first overtone, which is linked to the presence of methyl groups. It is known that methylation is an important step in the biosynthesis of lignins and has been shown to affect their properties (Louie et al., 2010). In a group of wavelengths that negatively contributed to OMD, signatures at  $\lambda = 1658\text{--}1662\text{ nm}$  could be related to the Ar-CH bonds in lignins.

Our study also highlighted  $\lambda = 1712$  and  $2328\text{ nm}$  as wavelengths of potential high importance for prediction of digestibility. These wavelengths were previously found to relate to the presence of hemicellulose and contributed negatively to calibrations predicting OMD (Schwanninger et al., 2011; Shenk et al., 2001). In the vicinity of these two wavelengths, we found that samples characterized by low OMD tended to have high absorbance values. Researchers analyzing the chemical composition of bamboo timber fractions have found that  $\lambda = 2328\text{ nm}$  is related to the C-H deformation and C-H stretching vibration of xylan. This wavelength is important for comparing young and mature bamboo timber, with higher absorbance expected

in older plants (Yang et al., 2016). Interestingly, we found that samples with higher NDF (and conversely, lower OMD) tended to have higher absorbance near these two wavelengths. Regarding other wavelengths contributing positively to the NDF calibrations,  $\lambda = 2310\text{--}2312\text{ nm}$  in the region of the C-H bend second overtone had a strong impact. These wavelengths have been found to be related to the presence of fats (Shenk et al., 2001). The authors of Decruyenaere et al. (2009) speculated that the relevance of fat wavelengths to digestibility might be related to the presence of cuticular wax of plant origin. Plant-wax n-alkanes have been used before as fecal markers of digestibility (Mayes & Dove, 2006). It has been shown that the total n-alkane concentration of perennial ryegrass decreases during the plant growth, making younger plants more abundant in n-alkanes than more mature individuals (Côrtes et al., 2005). However, the concentration of short-chain n-alkanes, which are found in the stem fraction of plants, increases in older swards, characterized by higher NDF values (McGovern et al., 2021). The wavelengths with negative regression coefficients impacting NDF prediction belong to regions previously linked to the presence of starch and sugars ( $\lambda = 886\text{ nm}$ ), cellulose ( $\lambda = 2276, 2278\text{ nm}$ ), water ( $\lambda = 1942\text{--}1944\text{ nm}$ ), hemicellulose, and cellulose ( $\lambda = 2386\text{--}2392\text{ nm}$ ) (Schwanninger et al., 2011; Toledo-Martin et al., 2018; Workman, 2000).

In summary, the analysis of important wavelengths indicates that the combination band region (around  $2000\text{--}2500\text{ nm}$ ), close to the infrared range, appears to be important for prediction of feed digestibility from NIRS, especially when considering NDF. This could be explored further with a view to developing calibrations using portable or harvester-mounted spectrometers that typically cover a narrower range of  $900\text{--}1700\text{ nm}$ .

### Phenotypes on parental plants can predict forage nutritive value in subsequent generations grown as competitive swards

Most modern perennial ryegrass breeding programs are focused on the development of cultivars with improved forage nutritive value. This may involve selecting plants with reduced NDF contents and/or increased OMD values to enhance animal production potential from pastures (Beecher et al., 2018; Kendall et al., 2009). The selection process typically takes place in large spaced-plant nurseries, where plants are evaluated for traits associated with forage nutritive value. Plants with the highest values are then crossed to produce full-sib families, half-sib families, or synthetic varieties, which are subsequently evaluated in-field for traits like forage yield that cannot be directly measured in SPs. Although, in this study, decisions on which plants to cross to obtain full-sib families were not based on their nutritive value, which was not yet measured at the time of pair-crossing (parents were chosen at random), this study aimed to determine how selections based on parental-SPs predicted the forage nutritive value of full-sib families.

In predicting the OMD and NDF of  $F_2$  families from parental midpoints derived from the SP data, our results showed that PA varied across cuts, with values of up to

0.61 for NDF and of up to 0.54 for OMD. The PA decreased substantially when the mean of the parental pool was used for prediction, which is unsurprising, given the significant variation within families for the target traits. Classifying  $F_2$  families according to the source of parental material, based on SPs family means, revealed a trend whereby families whose parents were sourced from families or cultivars with a mean for the target trait in the top 30th percentile tended to be situated at the extremities of the feather plots, specifically at the lower tip for NDF and the upper tip for OMD. However, due to the variation within SP families, in many  $F_2$  families originating from theoretically “top” parent material, the overall performance was average or even below average.

As several SP families and perennial ryegrass  $F_2$  families were derived by crossing with IBERS cultivars, we could analyze the distribution of digestibility in those families. In  $F_2$  families with some IBERS parentage, we observed no clear pattern for OMD, whereas a clustering was evident for NDF (Figures S10 and S11). This may be attributed to the fact that the IBERS plant breeding program was focused on selecting plants with high water-soluble carbohydrates (WSC), a trait that is strongly correlated with dry matter digestibility (DMD) and NDF (Colas et al., 2022; Robins & Alan Lovatt, 2016). The differences in distribution demonstrate that NDF and OMD cannot be used interchangeably as estimates of nutritive value. As suggested by Colas et al. (2022), NDF, which is related to cell wall content, is one of the underlying traits that contribute to OMD.

### Genomic models to accelerate genetic gain for forage nutritive value in perennial ryegrass breeding programs

The utilization of genomic-assisted breeding strategies will be important for boosting the rate of genetic gain in perennial ryegrass breeding programs for critical traits like forage nutritive value. This study reveals that the genomic prediction models developed for both OMD and NDF in a substantially sized SP training population showed moderate predictive capabilities, ranging from 0.43 to 0.57 for NDF across cuts and from 0.52 to 0.59 for OMD across cuts. Slightly higher PA values obtained for OMD might be partially explained by NDF being considered an underlying trait contributing to OMD, as was already mentioned earlier (Colas et al., 2022). Due to the population structure in SPs, shown by Byrne et al. (2017) in Figure 2, the obtained results from the random cross-validation might be higher than if the SPs came from an evenly mixed population (Guo et al., 2014).

The findings of this study are consistent with previous research on forage grasses, which has identified similar trends in the prediction of agronomic traits. Specifically, studies such as Arojju et al. (2020) have reported PAs of up to 0.38 for energy traits and 0.36 for fiber traits, based on multi-location data. This compares to our results obtained with LOO cross-validation in  $F_2$  families for NDF. Additionally, Fè et al. (2016) obtained a PA of 0.56 for NDF in a large population of  $F_2$  families, whereas Grinberg et al. (2016) obtained the highest PA

value of 0.44 for DMD, using prediction with genotyping data from mother-plants and phenotypic values obtained on competitive swards (with lower values obtained depending on the training population). A comparison of various modeling techniques yielded minimal differences between approaches using these data, which is consistent with previous research (Grinberg et al., 2016; Merrick & Carter, 2021).

Our analysis revealed that the PA of models developed using SPs was higher than those developed using  $F_2$  families. This difference may be ascribed to variations in sample preparation. In SPs, forage samples were carefully collected to exclude stems, ensuring that phenotyping would closely reflect leaf blade digestibility. Conversely, when sampling sward material from  $F_2$  families, samples were taken from the entire cut, including stems and pseudostems. Thus, the decrease in PA for NDF could be attributed to this disparity in sampling approaches, with NDF potentially being more susceptible to the change in procedure than OMD. After stem emergence, the overall nutritive value is affected by the presence of stems. Colas (2021) found that stem quality is generally lower than leaf quality and it decreases strongly with maturity. Our results suggest that OMD could be a more practical estimate of digestibility in maturing swards than NDF. OMD is not only explained by NDF but also by NDFD, which relates to the quality of the cell wall content (Baert et al., 2014). In the future studies, inclusion of NDFD for prediction of nutritive value could be considered.

Another likely explanation is that models developed using SPs have access to matching genotype and phenotype data from individual plants, whereas models developed using  $F_2$  families utilize mean phenotypes and mean genotypes. The use of family mean genotypes for genomic prediction in perennial ryegrass has already been demonstrated (Cericola et al., 2018; Fè et al., 2016; Pembleton et al., 2018). Additionally, the method of encoding  $F_2$  families genotypes might also have been a factor. We have chosen to categorize family SNP data into bi-allelic genotypic calls, as opposed to utilizing allele frequencies. While our decision has been dictated by the sequencing depth, this might have influenced the observed difference in PA. It is also worth noting that while an equivalent number of markers was used in the development of prediction models, the size of the training set was significantly larger in the case of the SPs. It is also expected that some of the PA in the SP population is arising from structure in this population and we did observe a decrease in PA when using LOFO cross-validation in the SP population. The  $F_2$  family population represents a more realistic breeding scenario.

Our results indicate that models trained on SPs can be used to predict phenotypic values in  $F_2$  families, although with lower mean PA than when predicting among SPs or among  $F_2$  families. However, the decrease in PA observed in our study might be overestimated, as we were able to use only the 5178 common SNPs, shared between the SPs and  $F_2$  family marker data sets. We have already discussed in Konkolewska et al. (2023) that the common SNPs are not randomly distributed on the chromosomes, and their use resulted in a general decrease in PA in multiple traits.

Additionally, we found that when the  $F_2$  family marker data set is reduced to 5178 randomly selected SNPs, the PA obtained when predicting nutritive value is comparable to results obtained on a full data set. This indicates that the small number of SNPs is most likely not the only limiting factor causing the observed decrease in PA. We expect that using the same genotyping approach to obtain SNPs for SPs and  $F_2$  families would result in higher PA when performing an empirical assessment, even when a comparably small data set of randomly selected markers (around 5000 SNPs) would be used.

When establishing a reference population for genomic prediction in perennial ryegrass, one option is to conduct all evaluations on the  $F_2$  families, without conducting any evaluations on individual SPs (Figure S12). Alternatively, genotypes could be obtained from individual plants that are also evaluated for certain traits (e.g., forage nutritive value) in SP field trials (Figure S13). These plants would serve as parents for developing  $F_2$  families for yield evaluations, with the selection candidates being plants from within the top-performing  $F_2$  families.

The results suggest that using a reference population consisting of individual plants leads to higher prediction accuracy (PA) compared to a reference population consisting of  $F_2$  families, albeit the decrease in PA was modest, especially considering that the reference population of  $F_2$  families was much smaller than the reference population of SPs. The advantage of using  $F_2$  families to build a reference population is that it eliminates the need for SP field trials, with all evaluation data collected in competitive swards. This would be particularly advantageous if forage nutritive value data could be captured while harvesting using harvested-mounted NIRS, providing these data without any extra work. However, it is still unclear how effectively harvested-mounted NIRS can be used to predict forage nutritive value parameters.

One potential application of GS in perennial ryegrass breeding is for within-family selection, enabling the best plants to be selected within the best families. In a scenario where parental plants are used for model training and the selection candidates are  $F_2$  individuals, it would require that PA be maintained over two rounds of recombination. While we do not have individual genotypes and phenotypes of  $F_2$  seedlings, we do have mean genotypes and phenotypes of  $F_2$  families. The SP and  $F_2$  families were genotyped using different restriction enzymes for complexity reduction before genotyping-by-sequencing. However, there was overlap in just over 5000 SNPs distributed across seven chromosomes (these SNPs have been described in Konkolewska et al., 2023). Using these SNPs enabled us to evaluate genomic prediction from SP to  $F_2$  families, which showed that while PA was lower than that obtained using cross-validation within the population of  $F_2$  families, it only decreased by 18% for NDF and 20% for OMD. This is encouraging for the application of among- and within-family GS in perennial ryegrass, which has already been shown in simulations to lead to acceleration in the rate of genetic gain (Barrett et al., 2021; Esfandyari et al., 2020).

Encouragingly, PA could be maintained with a reduced SNP set; hence, it is very likely that the development of

inexpensive genotyping approaches will be a core requirement for cost-effective GS in perennial ryegrass.

## CONCLUSIONS

The limited genetic gain for forage nutritive value in perennial ryegrass breeding, despite its significance for animal performance, is a challenge that must be addressed. With the aid of a substantial reference population of breeding material, we were able to demonstrate that there is ample phenotypic variation within breeding populations that can be utilized to improve forage nutritive value. Additionally, we found that mid-parent phenotypes collected from plants grown as individually SPs can effectively predict OMD and NDF in  $F_2$  families grown as competitive swards.

The accuracy of predicting OMD and NDF from genomic data is moderate, and the models developed on individual plants outperform those developed from family means. Furthermore, the genomic prediction models developed on parental plants can predict OMD with moderate accuracy in subsequent generations grown as competitive swards. These findings are encouraging and suggest that there is potential for genetic improvement in forage nutritive value through the application of genomic prediction models.

## AUTHOR CONTRIBUTIONS

**Agnieszka Konkolewska:** Data curation; formal analysis; funding acquisition; investigation; project administration; resources; software; visualization; writing—original draft; writing—review and editing. **Michael Dineen:** Methodology; resources; supervision. **Rachel Keirse:** Data curation; methodology. **Patrick Conaghan:** Conceptualization; funding acquisition; project administration; resources; supervision; writing—review and editing. **Dan Milbourne:** Conceptualization; funding acquisition; investigation; project administration; resources; supervision; writing—review and editing. **Susanne Barth:** Conceptualization; methodology; resources; supervision. **Aonghus Lawlor:** Resources; supervision; writing—review and editing. **Stephen Byrne:** Conceptualization; data curation; funding acquisition; investigation; methodology; project administration; resources; software; supervision; visualization; writing—original draft; writing—review and editing.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

Additional data and code are available in a Figshare repository (10.6084/m9.figshare.c.7128643).

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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## APPENDIX

### Chemometric modeling

Prior to calibration, the data set was limited to observations in the range of 800–2500 nm, to focus on the near infra-red region. Next, a ROBust PCA algorithm (Reynkens, 2018) were run to detect and remove samples falling outside both score and orthogonal distance cut-offs (i.e., bad leverage points). The resulting diagnostic plot is shown in Figure A1.

A number of spectral pretreatments was applied to improve signal quality before modeling. First, pretreatments to correct for light scatter noise and correct the baseline were performed. These included: multiplicative scatter correction (MSC), extended multiplicative scatter correction (EMSC), standard normal variate (SNV),

detrend, baseline, as well as combinations of treatments: SNV + detrend, MSC + detrend, SNV + baseline, MSC + baseline. The MSC correction was performed using the msc function (Mevik et al., 2020). Baseline correction was performed with the spc.fit.poly.below function (second order polynomial) (Beleites & Sergo, 2014). Detrend correction was performed with the detrend function (linear) (Borchers, 2021). EMSC correction was performed with the EMSC function (Liland, 2021). Second, mathematical derivative treatments were performed along with the Savitzky–Golay smoothing, using sgolayfilt function (Uwe et al., 2014). Three treatments were chosen: no mathematical treatment, first or second order derivative, with second filter order, and filter length of 7, which will be referred to as “0.7.2,” “1.7.2,” and “2.7.2” treatment, respectively. Three linear models have been considered:

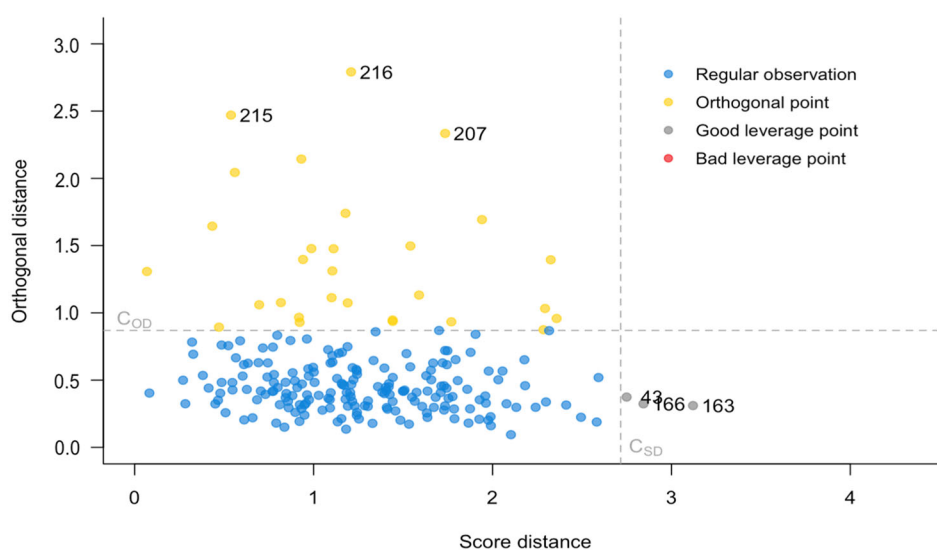


**FIGURE A1** Diagnostic plot for PCA with the score distances (SD) on the x-axis and the orthogonal distances (OD) on the y-axis, following an analysis on the calibration sample performed with robust PCA. Three samples with most extreme SD and OD are labeled with sample numbers.  $C_{OD}$ , orthogonal distance cutoff;  $C_{SD}$ , score distance cutoff.

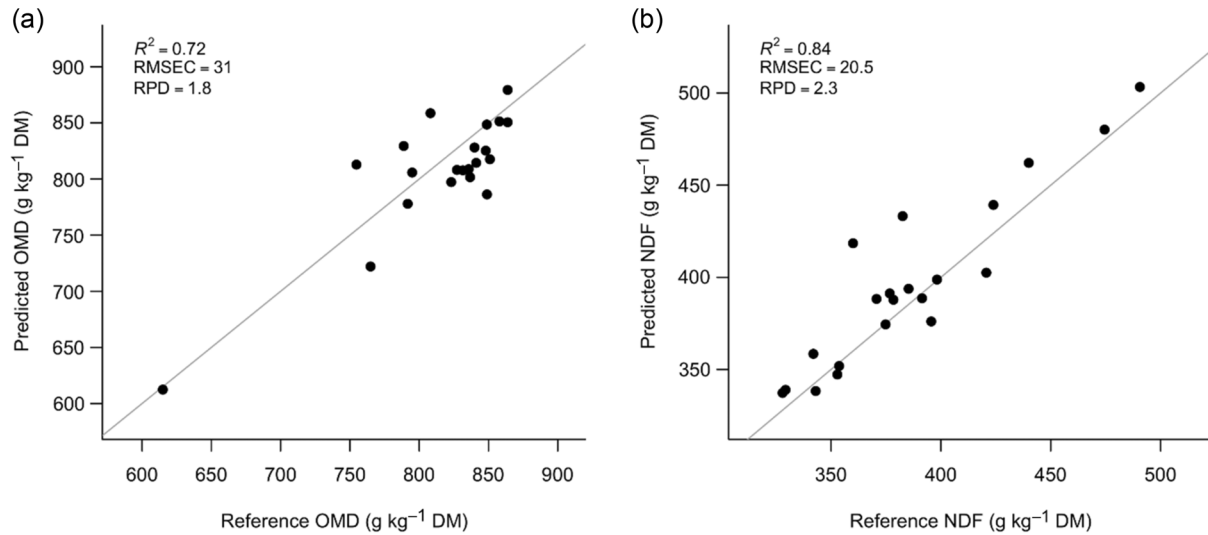


**FIGURE A2** Linear relationship between NIRS predicted and laboratory reference values for models tuned on the full calibration sample with optimum parameters chosen following Vall. Gray line marks the target line. NDF, neutral detergent fiber; NIRS, near-infrared reflectance spectroscopy; OMD, organic matter digestibility;  $R^2$ , coefficient of determination; RMSEC, root mean squared error of calibration; RPD, relative predictive deviation.

Partial Least Squares regression (PLS), Elastic net, and K-Nearest Neighbors Localized Weighted PLSR (KNN-LWPLSR, abbreviated to LWPLSR). For PLS, function *pls* package was used (Mevik et al., 2020), with scaling of data enabled. The Elastic net model was implemented with *glmnet* package (Friedman et al., 2010), with centering and scaling enabled. LWPLSR was implemented with *rchemo* package (Lesnoff, 2021). Data were split randomly to training and held-out test datasets ( $n = 198$ , 90%; and  $n = 21$ , 10%, respectively). A 10-k fold procedure was used for parameters tuning on the training data:  $\alpha$  (range: 0–1) and  $\lambda$  (range: 0–1) for Elastic net, number of principal components (range: 1–30) for PLS, and  $k$  (range: 10–50) and  $nlv$  (range: 1–10) for LWPLSR; where  $\lambda$  is the regularization strength parameter,  $\alpha$  is the ratio between  $L_1$  and  $L_2$  penalty, ranging from 0 (Ridge Regression) to 1 (Least Absolute Shrinkage and Selection Operator, LASSO),  $k$  is the number of nearest neighbors to select for each observation to predict,  $nlv$  is the number of latent variables to calculate in the local PLSR models. Outlier analysis was conducted on both the training and test data sets, with samples being farther than 3 standard deviations (SDs) from the mean per each trait ending removed. The optimal parameters and preprocessing strategies for each model were selected in terms of the lowest root mean squared error of prediction. Next, models were trained on a full training data set and used to predict on held-out test data. Models were evaluated on the basis of root mean squared error of prediction (RMSE), mean absolute percentage error (MAPE), relative predictive deviation (RPD), and range error ratio (RER), for the traits of interest (as defined in Equations a1–a5). Performance of the optimum models in prediction of forage nutritive value traits on the withheld data is shown in Figure A2.

### Statistical formulae

Let  $n$  be the number of data points,  $y_i$  be the observed laboratory reference value and  $\hat{y}_i$  be the NIRS calibration predicted value for the  $i$ th data point. Then, RMSE can be defined as

$$\text{RMSE} = \sqrt{\frac{\sum_{i=1}^n (y_i - \hat{y}_i)^2}{n}}. \quad (\text{a1})$$

MAPE can be defined as

$$\text{MAPE} = \frac{100\%}{n} \sum_{i=1}^n \left| \frac{y_i - \hat{y}_i}{y_i} \right|. \quad (\text{a2})$$

RPD, the ratio between the SD of the laboratory reference values and the standard error of prediction (SEP), can be defined as

$$\text{RPD} = \frac{\sigma}{\text{SEP}}, \quad (\text{a3})$$

where  $\sigma$  is the SD of the reference values and SEP the SD of the residual values corrected for bias, as defined by

$$\text{SEP} = \sqrt{\frac{\sum_{i=1}^n (\hat{y}_i - y_i - \text{bias})^2}{n - 1}} \quad (\text{a4})$$

with bias as defined in

$$\text{bias} = \frac{\sum_{i=1}^n (\hat{y}_i - y_i)}{n}. \quad (\text{a5})$$