IDENTIFYING MOLECULAR MARKERS ASSOCIATED WITH QUALITY AND QUANTIFYING THEIR POTENTIAL TO INCREASE ALFALFA VALUE

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ABSTRACT

Dairy producers purchase feed to meet protein, energy, and fiber, requirements for their cows and alfalfa is a viable feedstuff that contributes to those nutrient needs. NIFA funded research on first cut alfalfa in 2018 at Prosser, WA, Union, OR, and Kimberly, ID evaluating 200 diverse alfalfa accessions ranging in fall dormancy from 2 to 6. Forage quality analyses were conducted and calculated. Nutrient values were determined using information from Northwest United States from November 2017 to August 2019 to determine dollar value Mg^{-1} of first cut alfalfa. Forage quality traits were analyzed using empirical best linear unbiased prediction (EBLUP) and covariate was used for fall dormancy variation. Optimum forage quality constituents found in the trial were placed one at a time into protein, energy, fiber, and fiber fill calculations to determine dollar values to dairy nutrition. Greatest potential to increase dollar per ton hay value followed sequentially in order of importance by: increasing metabolizable protein, decreasing ash content, decreasing lignin, increasing fat, decreasing acid detergent insoluble crude protein, and increasing neutral detergent insoluble crude protein. If all constituents were maximized, it increased in alfalfa hay from an average value of $274 to $364 / ton for an increase of $90/ton. This data was also used for marker-trait association analysis. Ninety-one significant genetic markers were found on 21 traits on the first year data. Markers that were consistently significant across multiple traits, including: aNDF, RFV and Energy NRC 2001 Lignin. After validation, these markers can be used for marker-assisted breeding to improve alfalfa quality.

INTRODUCTION

Forage quality is a primary concern that is shared in the industry, as evidenced by surveys of forage specialists, alfalfa growers, and alfalfa consumers (Ottman et al., 2013; Washington State Hay Growers Association, 2017). The importance of maintaining forage quality is not only important for downstream feed applications in animal health, but is also a key distinguisher during times when the demand for the product is less than the available supply. As of now, the use of genetic information to directly improve forage quality has been largely under-utilized in alfalfa. A list of measurable components of fiber quality can be found in Table 1. If these

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advances were available, breeding programs could be established that improve forage quality, increase farm-gate values, and enhance the economics of alfalfa production in the Pacific Northwest (PNW) and other major growing regions throughout the US. The *overall goal* of this project is to achieve these outcomes: **1)** quantify the genetic diversity in forage quality that exists in a diverse collection of 200 alfalfa varieties; **2)** quantify the relationship between alfalfa quality and other possible confounding and important agronomic parameters in these varieties; and **3)** identify genetic information associated with forage quality to foster productive breeding programs that select for quality.

Table 1. Laboratory measurements of fiber related to forage quality

Item	Definition
NDF	Neutral detergent fiber. Lab assay that measures total cell wall content of plant material. Composed primarily of cellulose, hemicellulose and lignin. NDF affects animal performance by restricting feed intake and reducing energy value (TDN) of forage
iNDF	Proportion of NDF that is indigestible in ruminants. Measured as the NDF remaining after long-term (240 h) in vitro incubation in rumen fluid. **We hypothesize that the iNDF component of fiber is affected in part by biosynthesis of lignin, cellulose, and hemicellulose.**
pdNDF	Potentially digestible NDF. Calculated as pdNDF= NDF-iNDF. Proportion of plant fiber that can be digested by bacteria. The absolute amount of pdNDF digested depends on its rate of digestion and time exposure to fiber digesting bacteria.
kd	Rate of digestion of pdNDF. Measured by calculating the change in pdNDF remaining after 24, 30 and 48 hours of incubation in rumen fluid. **We hypothesize that the rate of fiber digestion is influenced by the biosynthesis of lignin, cellulose and hemicellulose**.
NDFD	NDF digested after a defined period of time. Example NDFD48 is the measured proportion of total NDF remaining after 48 hours incubation in rumen fluid. Typical incubation time measurements are 24, 30, 48 and 240 hours.
TTNDFD	Total tract NDF digestibility. A calculated estimate of in vivo NDF digestion in ruminants. TTNDFD integrates pdNDF, kd and rate of passage into a single digestibility coefficient for NDF.

<u>The goal of this research includes four objectives</u>:

1) Determine quality and stage of maturity at first harvest of 200 alfalfa varieties at three locations in the PNW. Fall dormancy and yield for all cuttings will be determined to identify and reduce confounding factors with quality.

2) Quantify the genetic diversity of alfalfa that is related to forage quality to understand the potential to breed new alfalfa varieties for higher forage quality.

3) Identify molecular markers associated with forage quality (NDF, NDFD24, NDFD30, NDFD48, kd, iNDF, TTNDFD) traits in alfalfa to aid alfalfa breeders.

4) Extend the knowledge gained from project to positively impact alfalfa producers, breeders and others in the alfalfa industry.

MATERIALS AND METHODS

Two hundred alfalfa genotypes were selected with fall dormancies ranging from one to six from diverse germplasm including 148 accessions from USDA-ARS Plant germplasm Introduction and Testing Research Unit collected from around the world. The remaining balances were 52 varieties from private alfalfa breeding programs including: S&W Seed Co., Corteva-Agriscience, Legacy Seeds and Blue River Hybrids. Three locations in PNW were selected for production including: Prosser, WA, Kimberly, ID and Union, OR in an augmented statistical design using Vernal and Alforex Hi-Gest 360 as checks in 11 blocks per location. DNA was extracted and genotyped by sequencing at the Center of Genome Research and Biocomputing, Oregon State University. Samples of first cutting harvested at the mid-bud stage was analyzed for quality traits including: NDF, NDFD24, NDFD30, NDFD48, kd, iNDF, TTNDFD and other traits using NIRS in the Rock River Laboratory, Watertown, WI 53094. Data was analyzed by a modified augmented design using fall dormancy as a covariate to eliminate its effect and using empirical best linear unbiased prediction (EBLUP). The EBLUP results were subjected for further analysis as mentioned in the results and discussion. To determine the potential value of the increased hay quality, methods from Dairy NRC 2001 for energy, metabolizable protein and fiber were used as well as fiber fill adjustment described by Weiss (2017, Western Alfalfa and Forage Symposium). Values used in the calculations came from Dec. 2017 to Aug. 2019 values (Tebbe & Weiss) from typical dairy feeds for PNW and averaged results are: Net Energy for Lactation (NE_L) \$0.11 \$$Mcal^{-1}$, metabolizable protein \$0.35 \$ lb^{-1}, effective fiber \$0.07 $lb^{-1}$and non-effective fiber \$-0.08 lb^{-1}. Adjustment for fiber fill at a milk price for \$18/cwt is +/- \$5.00 per aNDF unit from 44%. To determine genetic areas and molecular markers related to quality, significant markers was selected by using both General Linear Model (GLM) and Mixed Linear Model (MLM). In MLM analysis, the –log p–value was greater than 3.5; In GLM analysis, the –log p–value of each significant marker was greater than 5, and all the significant markers passed Benjamini-Hochberg correction test with the false discovery rate (FDR) of 0.05. The markers that met the above standards in both GLM and MLM were selected as the significant markers (Figure 3.).

RESULTS AND DISCUSSION

First year results show that significant increases TTNDFD and RFQ are still possible even after fall dormancy influence was removed from the data as shown (Figures 1 and 2). The vertical bars show the range in quality with the checks (Hi-Gest 360 and Vernal) being labeled. Hi-Gest 360 quality is above Vernal at all three locations and Vernal is near the average of all the plant introduction quality. However, at all locations, a significant increase in digestibility (TTNDFD) and Relative Feed Quality is still available.

If markets adopted this method of evaluating alfalfa, alfalfa would likely be fed as a larger portion of the diet as animal producers would see they are not just buying energy. The dollar per ton values are much higher than what hay is trading for today. Our results show the majority of value from alfalfa comes from energy, followed closely by protein (Table 2.). All sources of value must be included to get a proper value of alfalfa. These results show that if through breeding you were able to use the best and worst variety for protein, energy, fiber, and fiber fill from those we planted into one variety, the hay from this first cutting at mid bud stage alfalfa hay could have been worth as high as $330 /ton and as low as $236 /ton. This constitutes a range of $94 / ton, just based off of quality genetics inside this experiment, not including fall dormancy which is limited by environment. The potential range of nutrient values based off our germplasm was: $56.11, $32.93, $17.07, $10.88 above the average variety in this experiment for fiber fill adjustment, protein, energy and fiber, respectively. Fiber fill adjustment in this model is based off of aNDF, so aNDF is very important. Genetics used in this study, which in part came from the USDA Germplasm Repository for alfalfa, was from many different countries around the world and provides a good estimate of what is available in the alfalfa gene pool world-wide. Breeding that decreases neutral detergent fiber had the greatest potential to increase dollar per ton of hay, followed sequentially in order of importance by: increasing metabolizable protein, decreasing ash content, decreasing lignin, increasing fat, increasing neutral detergent crude protein and decreasing acid detergent insoluble crude protein. If all of these constituents were maximized the increase in alfalfa hay quality would be over $60 / ton over the average variety in our trial. One of the challenges in forage quality is the impact of changing one quality constituent has on all the constituents. This data shows as aNDF is decreased it increased protein, decreased lignin, and increased NDICP which is beneficial (Table 2.). A decrease in aNDF or increase protein content it results in an increase ash content, decrease fats content and ADICP is increased which hurts quality. If ash was increased, fat content decreased and ADICP increased the levels received in this trial the impact would only be $4.33 / ton (Table 3.). Which is minimal as compared to the value of minimizing aNDF and maximizing protein which combined is would be valued at $62.26/ton.

Analysis of the DNA and quality data from the first year revealed 91 significant genetic markers were found across 21 constituants. Molecular markers that are consistently significant across both General Linear Model (GLM) and Mixed Linear Model (MLM) include: aNDF, RFV and Energy NRC 2001 Lignin (Figure 3). Since aNDF has such a large impact on hay quality and hay is currently sold by RFV (which includes aNDF in its calculation) these are important findings. This data suggest that significant increases in alfalfa quality is possible with the aid of marker assisted breeding programs for alfalfa quality and they can have a possible maximum impact of raising the average variety’s value from $276/ton to $339/ton. Second year data is currently in process of being analyzed. Yield data from this study was collected but not discussed here. A second NIFA study for 2020 and 2021 has been approved on these research plots to look at yield and protein production and digestion in the dairy cow as well as develop NIRSC methods to measure them.

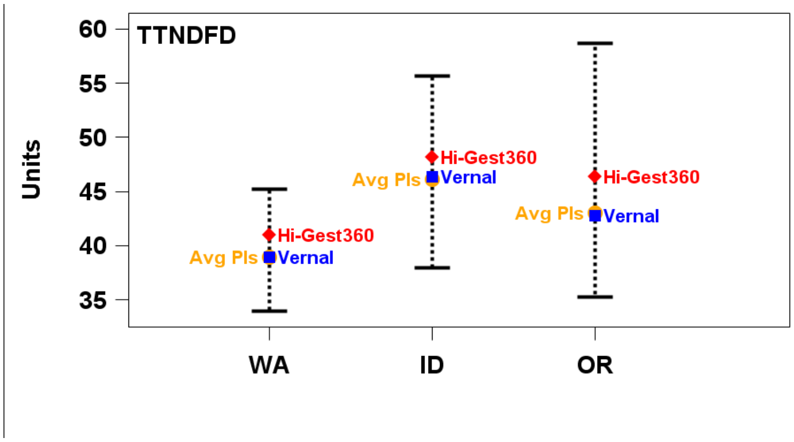

Figure 1. Total Tract Neutral Detergent Fiber Digestibility of forage from 150 plant introductions (PI) and 48 varieties as compared to the checks (Hi-Gest 360, and Vernal) grown at three PNW locations. Black lines show the range of responses and the average of PI is also given.

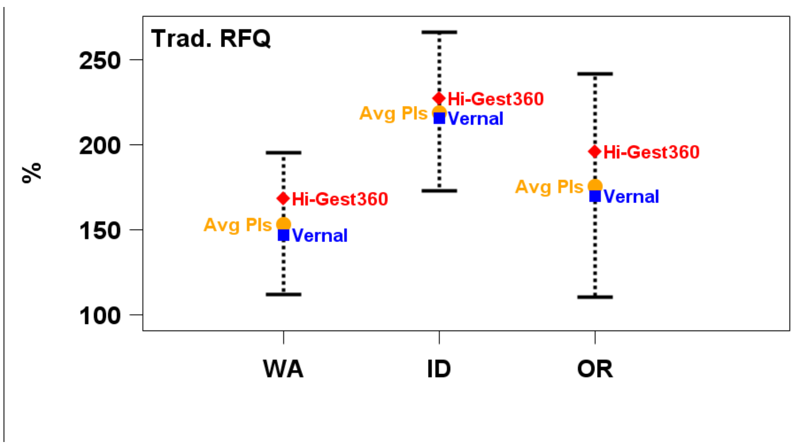

Figure 2. Relative Feed Quality (RFQ) of forage from 150 plant introductions (PI) and 48 varieties as compared to the checks (Hi-Gest 360, and Vernal) grown at three PNW locations. Black lines show the range of responses and the average of PI is also given.

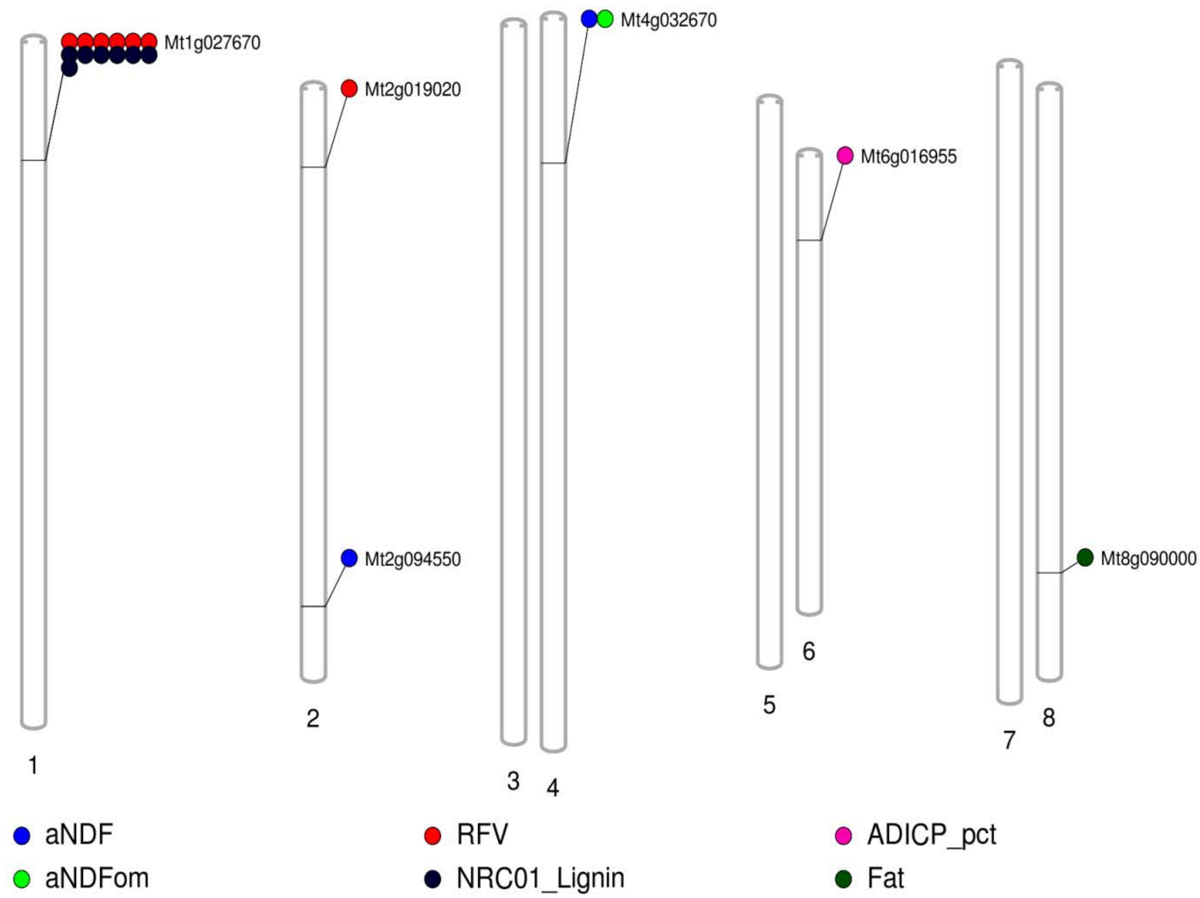

Figure 3. The eight significant markers associated with quality traits were identified by both General Linear Model (GLM) and Mixed Linear Model (MLM). The genetic locations of the associated markers (genes) are labeled on their respective chromosomes 1, 2, 4, 6 and 8 (out of total 8 chromosomes).

Table 2. The range of values received from first cut forage from the 200 alfalfa mixed cultivars and plant introductions used in the study including values for protein, energy, fiber, quality adjustment for fiber fill and total value for dairy cows.

Statistic	Protein Value	Energy Value	Net Fiber Value	Quality Adjustment	Total
	($/ton)	($/ton)	($/ton)	($/ton)	Hay Value ($/ton)
Maximum	$100.65	$132.50	$40.33	$68.15	$330.09
Minimum	$67.72	$115.43	$29.45	$12.04	$236.10
Average	$82.37	$123.43	$36.09	$33.90	$275.80
Range	**$32.93**	**$17.07**	**$10.88**	**$56.11**	**$93.99**
Standard Deviation	$11.21	$4.78	$3.82	$19.72	$31.33

Note: Values are based on Dec. 2017 to Aug. 2019 values from typical dairy feeds for PNW (Tebbe & Weiss) which are: Net Energy for Lactation $0.11 $Mcal^{-1}, Metabolizable Protein $0.35 $ lb^{-1}, Effective fiber $0.07 lb^{-1} and Non-effective fiber $-0.08 lb^{-1}. Adjustment for Fiber Fill at a Milk price for $18/cwt is +/- $5.00 per aNDF unit from 44%.

Table 3. First cut quality data over 200 diverse varieties with covariance for fall dormancy taken out, averaged over three locations including: Kimberly, Idaho; Union, Oregon; and Prosser, Washington and change in value per ton from average value to high value and total value using PNW values.

Constituents	Unit	Avg.	Low	High	Standard Deviation	Increase over the average variety ($/ton)	Total Value with increase $/ton
aNDF	%	37.22	30.37	41.59	3.94	$33.51	$309.09
Crude Protein	%	24.08	19.80	29.43	3.28	$22.96	$298.54
Ash	%	10.16	8.98	11.49	0.97	$2.86	$278.44
Lignin	%	6.41	5.59	7.20	0.39	$2.80	$278.38
Fats	%	2.00	1.69	2.24	0.15	$0.90	$276.48
NDICP	% of D.M	0.91	0.72	1.19	0.12	$0.23	$275.81
ADICP	% of D.M.	0.52	0.47	0.60	0.03	$0.17	$275.75
All constituents optimized						$63.20	$338.78

Table 4. Correlations between quality constituents (r values) with covariate data. Correlations that are antagonistic with selecting for aNDF and crude protein are in red. All correlations were significant at PR>0.001 .

	aNDF	Crude Protein	Ash	Lignin	Fats	NDICP	ADICP	Value per Ton
aNDF	1.000	-0.934	-0.987	0.937	0.639	-0.792	-0.192	-0.984
Crude Protein	-0.934	1.000	0.927	-0.950	-0.362	0.923	0.507	0.982
Ash	-0.987	0.927	1.000	-0.928	-0.645	0.783	0.213	0.971
Lignin	0.937	-0.950	-0.928	1.000	0.399	-0.852	-0.389	-0.852
Fats	0.639	-0.362	-0.645	0.399	1.000	-0.184	0.485	-0.505
NDICP	-0.792	0.923	0.783	-0.852	-0.184	1.000	0.646	0.870
ADICP	-0.192	0.507	0.213	-0.389	0.485	0.646	1.000	0.350
Value per Ton	-0.984	0.982	0.971	-0.852	-0.505	0.870	0.350	1.000

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