Classification of Single- and Double-Mutant Corn Endosperm Genotypes by Near-Infrared Transmittance Spectroscopy

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ABSTRACT

A total of 1,176 grain samples representing 10 different single- and double-mutant genotypic classes of specialty starch corn were used for developing various classification models based on near-infrared transmittance spectra. The genotypes used included amylose-extender (*ae*), dull (*du*), sugary-2 (*su2*), waxy (*wx*), *ae wx*, *ae du*, *ae su2, du wx* and *du su2*. Two-class classification models (only two genotypes compared) were developed using partial least squares analysis (PLS) while three-way and multiclass models were examined using principal component analysis (PCA). The effectiveness of the calibrations was evaluated by examining the percentage of unknown grain samples incorrectly classified. In general, two-class models performed better than multiclass models. However, they did not show improvement when discriminating among genotypes with overlapping amylose contents such *ae du* vs. *ae* and *ae su2* vs. *ae*. Three-way models including double-mutants and their corresponding single-mutant counterparts had misclassification percentages typically <5% using 14 PCA factors but again, with the exception of models including genotypes with overlapping amylose contents such as *ae du* vs. *ae* vs. *du*. The best multiclass model using all 10 genotypic classes simultaneously revealed only two classes (*ae su2* and *du*) with misclassification rates >10% based on 16 PCA factors. This study demonstrates that, depending on the material to be considered, near-infrared transmittance spectroscopy could be useful when segregation of specialty starch hybrids grain from other grain types is necessary.

Corn (*Zea mays* L) genotypes possessing single- and double-mutant combinations of recessive alleles that influence starch structure have been of continuing interest because of the novel properties they possess for food and nonfood uses (Katz 1991). If the demand for these specialty starches increases, improved methods may be required during grain storage and transport to ensure proper classification to avoid contamination. There exist several methods to distinguish among normal and various specialty starch genotypes based on visual phenotypes and other physical properties (Garwood and Creech 1972, Obanni and BeMiller 1996). These methods might not always be well suited for the purpose of quality control as they require training and considerable sample preparation. It was suggested in a previous study that near-infrared transmittance spectroscopy (NITS) could be used to rapidly identify specialty starch grain in corn by determining characteristic amylose contents of specific mutant genotypes (Campbell et al 1999). Although it was demonstrated that these quantitative models were possible, they did not always indicate the specific genotype of the sample due to overlapping ranges of amylose contents normally observed among these classes.

It has been demonstrated that qualitative models based on near-infrared spectra can be used for discriminating among unique grain types resulting from qualitative genetic effects. For example, work by Delwiche et al (1999) demonstrated that a partial least squares (PLS) model using diffuse reflectance spectra of wheat flour could be used to discriminate between normal grain and grain possessing wheat-rye chromosomal translocations with misclassification rates as low a 0%. In another study by Wang et al (1999a), numbers of dominant R alleles influencing kernel color in wheat were determined using classification models with misclassification rates as high as 22% with a four-class model and as low as 0% using a two-class model.

The purpose of this study was to determine whether similar classification calibrations based on NITS spectra could be used to identify various single- and double-mutant genotypes of corn with five specific objectives. Our first objective was to examine a multiclass NITS calibration for placing unknown samples into 10 different genotypic classes. Secondly, since normal, waxy (*wx*) and amylose-extender (*ae*) grain represent the three genotypic classes handled in the largest volume in the United States, our next objective was to narrow the analysis to two- and three-class calibrations for discriminating among these. Thirdly, two-class models were examined to discriminate all other single- and double-mutant classes from normal. The fourth objective was to discriminate double-mutant genotypes from their single-mutant counterparts, which may be useful for backcross conversion breeding programs. And finally, a two-class model was examined for *ae* genotypes to determine whether grain samples with high starch amylose levels could be discriminated from those with lower values.

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MATERIALS AND METHODS

Genetic Materials

Genetic materials included in this study were obtained from breeding nurseries located near Lafayette, IN (1995), Kirksville, MO (1997), and Ponce, Puerto Rico (1997). The material represented many public inbred lines (B73, B94, B37, W64a, A634, Mo17, H99, Oh43, H111, Pa91) and hybrid combinations of these inbreds that had been converted to single- and double-mutant combinations of recessive alleles influencing starch structure. In addition, inbred and hybrid grain samples were obtained from various private seed companies including the Plant Genetics Group of National Starch and Chemical Co. (Indianapolis, IN) which supplied many of the high-amylose genotypes. A total of 1,176 individual ear

TABLE I
Numbers of Bulk Grain Samples Used for Each Single- and Double-Mutant Genotypic Class Included in the Calibration Set to Establish Classification Models and Validation Sets to Test Models

Genotypic Class[a]	Calibration Set (*n*)	Validation Set (*n*)
ae wx	46	47
ae su2	54	54
du wx	49	50
su2	49	50
du	25	26
ae du	59	59
du su2	77	78
wx	53	53
ae	106	106
Normal	67	68
Total	585	591

[a] Amylose-extender (*ae*), dull (*du*), sugary-2 (*su2*), waxy (*wx*).

samples were used that fell into 10 single- and double-mutant genotypic classes: normal, amylose-extender (*ae*), dull (*du*), sugary-2 (*su2*), waxy (*wx*), *ae wx*, *ae du*, *ae su2, du wx,* and *du su2*. Means and ranges of apparent amylose values for each genotypic class were determined using a colorometic iodine binding technique described by Campbell et al (1999). Grain from individual ear samples was dried to a moisture content of ≈12–13% and used for NITS scanning. Samples within each genotypic class were randomly assigned to a calibration and validation set (Table I). Calibration samples were used for development of PLS and principal component analysis (PCA) models while those used in the validation set were used to evaluate the models.

NITS Scanning

NITS spectra were collected from all grain samples using an Infratech 1255 grain analyzer (Foss North America, Eden Prairie, MN). A sample spectrum consisted of averaging the scans of 10 subsamples obtained from the grain of an individual ear of corn. Spectra (log [1/T]) collected from each subsample ranged from 850 to 1,048 nm in 2-nm increments and were collected from ≈20 g of grain filled level in the instrument's sample cups. When the amount of grain was limited, the sample was repoured into the sample cups until the desired number of subsamples was achieved.

Development of PCA Discriminate Calibrations

PCA was used to generate two-, three- and multiclass calibrations. This allowed an unknown sample to be assigned to a proper genotype when two or more genotypic classes were considered simultaneously. NITS spectra from the calibration samples of each genotypic class were subjected to PCA using the Unscrambler program (Camo A/S V6.11, Trondheim, Norway). The PCA analysis was repeated using varying numbers of principal component factors or eigenvectors (5, 6, 7, 8, 9, 10, 12, 14, 16) to create class-models for each genotype to statistically define each genotypic class based on possible unique features of their spectra. Unknown grain samples, represented by the validation set, were then assigned to the appropriate genotypic class using Soft Independent Modeling of Class Analogy (SIMCA) (Martens and Naes 1989). With this method, a sample was determined to belong to a specific genotypic class if it has the smallest sample-to-model distance (S_i). The distance from validation sample *i* to class-model *m* was calculated as the orthogonal distance from the sample to the different class-models as defined by their principal components. The distance (S_i) of validation sample (i) to the class-model (m) was computed as: $S_i(m,i) = \sqrt{ResXCal_{new,m}(a,i)}$ where $ResXCal_{new,m}$ is the residual variance per sample in *X* validation samples with *a* principal components for *i* sample numbers. The performance of a model was evaluated by determining the number of unknown samples that were misclassified (i.e., placed to a wrong genotypic class). The best discrimant calibrations were those with a misclassification value of 0 and the lowest number of principal component factors.

Development of PLS Discriminant Calibrations

Only two-class models were constructed using PLS analysis because the genotypic classes had no continuos nature with regard to physical or chemical properties. Unscrambler was used again for PLS, where sample spectra of the two genotypes to be compared were respectively coded as either "1" or "2". Validation samples with predicted code values <1.5 were assigned to the geno-

TABLE II
Multiclass Principal Component Analysis (PCA) Calibrations for Discrimination of Single- and Double-Mutant Genotypes Evaluated by % of Validation Samples Misclassified

	% Misclassified Using Multiple Factors						
Genotypic Class[a]	7	8	9	10	12	14	16
ae wx	38.3	14.9	17.0	17.0	2.1	8.5	4.3
ae su2	33.3	35.2	35.2	22.2	38.9	38.9	38.9
du wx	26.0	2.0	6.0	6.0	0.0	0.0	0.0
su2	68.0	46.0	44.0	44.0	34.0	20.0	10.0
du	26.9	30.8	23.1	19.2	3.8	38.5	38.5
ae du	44.1	28.8	28.8	25.4	18.6	15.4	10.2
du su2	48.7	26.9	19.2	9.0	16.7	15.3	9.0
wx	24.1	5.6	7.4	1.9	3.7	0.0	5.7
ae	33.3	28.6	27.6	38.1	22.9	12.4	5.7
normal	7.4	1.5	4.4	4.4	4.4	4.4	2.9

[a] Amylose-extender (*ae*), dull (*du*), sugary-2 (*su2*), waxy (*wx*).

TABLE III
Three-Class Principal Component Analysis (PCA) Calibrations and Two-Class Partial Least Squares Analysis (PLS) Calibrations for Discrimination of *wx, ae,* and Normal Corn Genotypes Evaluated by % of Validation Samples Misclassified

	% Misclassified Using Multiple Factors							
Genotypic Class[a]	5	6	7	8	9	10	12	14
PCA								
ae vs. *wx* vs. normal								
ae	5.7	7.6	9.5	0.0	1.0	0.0	0.0	0.0
wx	24.5	22.6	18.9	3.8	5.7	1.9	1.9	0.0
normal	7.4	8.8	0.0	0.0	4.4	4.4	2.9	2.9
PLS								
ae vs. normal								
ae	9.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
normal	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
normal vs. *wx*								
normal	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
wx	1.8	1.8	1.8	0.0	0.0	0.0	0.0	0.0
ae vs. *wx*								
ae	3.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
wx	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a] Amylose-extender (*ae*) and waxy (*wx*).

typic class coded by 1 and those samples with predicted values >1.5 were assigned to the class coded by 2. Models were examined across a number of PLS factors and were evaluated by the number of validation samples misclassified.

RESULTS AND DISCUSSION

Multiclass Calibrations

A multiclass PCA calibration that included all 10 genotypic classes was first examined. Misclassification rates using varying numbers of principal component factors are shown in Table II. Of all discriminant calibrations, it was expected that the multiclass model would be least strong because it must account for the uniqueness of all genotypic classes compared with calibrations including fewer classes. Of the 10 genotypic classes, the genotypic classes that appeared to possess the most unique spectra were *du wx*, *wx,* and normal because misclassification rates for the three classes were <10% with as few as eight factors. Upon the inclusion of more factors, the number of classes with misclassification rates of ≤10% increased. For example, at 12 factors, half of the classes had misclassification rates of <5%. However, even with 16 principal components, misclassification rates for the genotypes *ae su2* and *du* were relatively high (38.9 and 38.5%, respectively).

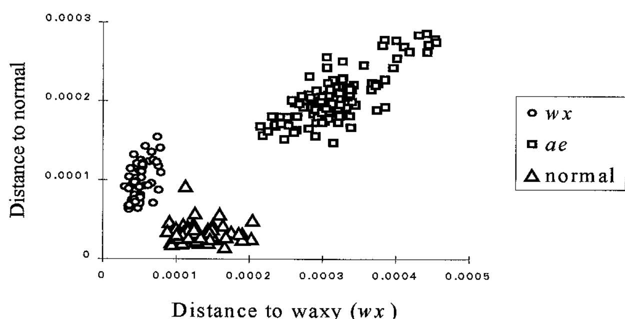

Fig. 1. Coomans plot showing distance of normal, *wx,* and *ae* validation samples from *wx* and normal calibration models.

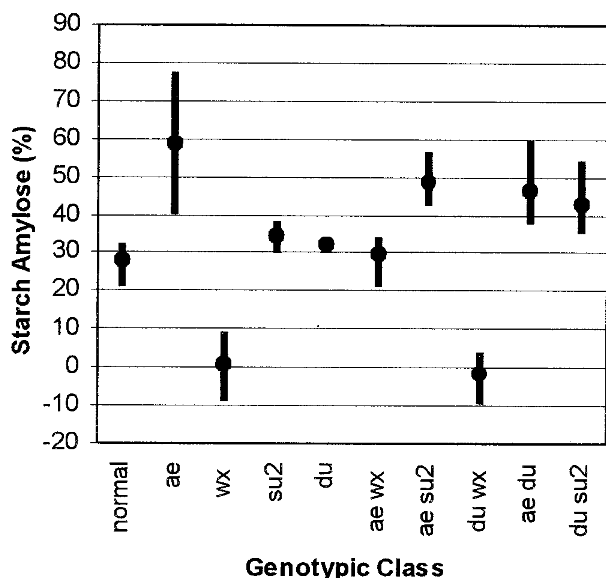

Fig. 2. Summary of mean, minimum, and maximum starch amylose values for each of 10 genotypic classes.

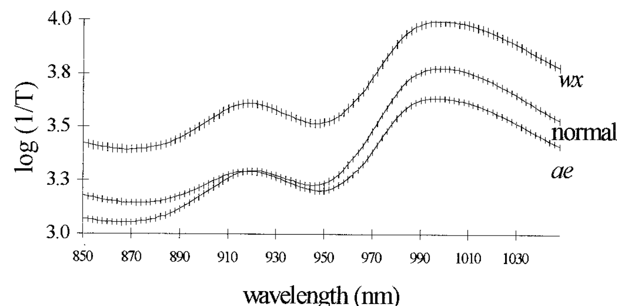

Fig. 3. Mean (± standard error) transmittance spectra of all normal, *wx,* and *ae* bulk grain samples.

Comparison of Normal, *wx*, and *ae*

Discriminate calibrations using only grain samples from normal, *ae,* and *wx* genotypes based on PCA and PLS are shown in Table III. As mentioned previously, it was expected that three-class PCA calibrations would out-perform a multiclass model because there were fewer classes for unknowns to be wrongly classified into, which turned out to be the case. For example, using only eight PCA factors resulted in misclassification rates at <5% of the three genotypes. In addition, a Cooman's plot showing the orthogonal distances from the validation samples to the *wx* and normal models clearly illustrates the discrete grouping of the three genotypes by PCA (Fig. 1).

Misclassification rates of two-class PCA and PLS calibrations were also examined for normal, *wx,* and *ae* genotypes (Table III). For these genotypes and all other genotypes used in this study, two-class PLS calibrations out-performed two-class PCA calibrations in every case; therefore, two-class PCA results are not reported. Compared with the three-class calibrations, misclassification rates were minimized using fewer numbers of factors. For example, at a factor level of six, each of the three comparisons representing all combinations of *wx*, *ae,* and normal resulted in rates of 0%, with only one exception.

In a previous study by Campbell et al (1999), quantitative models based on PLS discriminated among *wx*, normal, and *ae* genotypes based on difference in starch amylose content. These three genotypes are clearly distinct from one another based on nonoverlapping amylose levels as shown in Fig. 2. Therefore, the three genotypes were compared to determine whether distinct differences in starch amylose contents were associated with differences in their spectra (Fig. 3). From the spectra, it was apparent that the amount of radiation transmitted through the sample at 950–1,050 nm was greatest for the *wx* genotypes followed by normal and high-amylose grain, respectively. The uniqueness of the sample spectra is not likely the direct result of differences in starch structure but is likely the result of differences in opacity of the samples.

Normal vs. Other Single- and Double-Mutant Genotypes

Two-class PLS calibrations for discriminating normal grain from various other single- and double-mutant genotypes are shown in Table IV. It was expected that these calibrations would be strong due to large physical differences that are typically observed visually as reported by Garwood and Creech et al (1972). This was indeed the case because misclassification rates were generally low with only five factors and without exception decreased to zero with nine factors.

Double-Mutants vs. Single-Mutant Counterparts

As discussed previously, single- and double-mutant genotypes are usually visually distinct from normal grain; however, differences among some genotypes may be less obvious. Therefore, three-way PCA calibrations were examined for five double-mutant genotypes and their single-mutant counterparts (Table V). Two of the best-performing calibrations were of the double-mutant *du wx* and *ae wx* and their respective single-mutant genotypes with misclassification at or near 0 with 12 principal components. Another calibration with a slightly less predictive capability was that of *du su2* and its respective single-mutant counterparts where, at 14 principal components, misclassification rates for the three genotypes

were ≤6%. Poor discrimination was made with calibrations including the double-mutants *ae du* and *ae su2* because misclassification of one member was >20%. As previously observed, the strength of models appears to be dependent on class differences in amylose content. For example, when *wx* samples were compared with nonwaxy sample, discrimination was improved. Conversely, when classes such as *ae* and *ae su2*, which have overlapping ranges of amylose content (Fig. 2) were compared, discrimination worsened.

To improve the resolution between some of the genotypes, two-class PLS calibrations were constructed and evaluated (Table VI). In nearly every case, discrimination among genotypes improved according to the low misclassification rates at lower factor levels. The one exception was the comparison between *ae su2* and *ae*, where misclassification rates generally remained high, 14.8 and 15.8%, respectively.

High- vs. Low-Amylose Genotypes Within *ae*

Large variations in amylose content due to quantitative genetic effects are observed within the ae genotype (Fergason 1994). Although considered as quantitative variation, in commercial settings, high-amylose corn is categorized under names such as Amylomaize V and Amylomaize VII which designate grain with ≈50 and 70% starch amylose levels, respectively. For the purpose of this study, *ae* genotypes were divided into two groups. A "low" group was assigned to samples with amylose levels of 46–57% while those designated as "high" had amylose levels of 59–76%. Apparent amylose was determined using a colorometric iodine binding method described in a previous study (Campbell et al 1999). With samples forced into these discrete groups, a two-way PLS calibration was examined; misclassification rates for the two groups are shown in Table VII. Misclassification rates were relatively high with rates beyond seven factors of ≈30 and 10%, respectively, for the low and high groups.

TABLE IV
Two-Class Partial Least Squares Analysis (PLS) Calibrations for Discrimination of Various Single- and Double-Mutant Genotypic Classes from Normal Corn Evaluated by % of Validation Samples Misclassified

Genotypic Class[a]	% Misclassified Using Multiple Factors				
	5	6	7	8	9
su2 vs. normal					
su2	8.0	6.0	2.0	0.0	0.0
normal	2.9	0.0	0.0	0.0	0.0
du su2 vs. normal					
du su2	1.2	0.0	0.0	0.0	0.0
normal	5.8	2.9	0.0	0.0	0.0
ae du vs. normal					
ae du	16.9	3.4	0.0	0.0	0.0
normal	2.9	0.0	0.0	0.0	0.0
du vs. normal					
du	3.8	3.8	0.0	0.0	0.0
normal	0.0	1.4	1.4	1.4	0.0
du wx vs. normal					
du wx	0.0	0.0	0.0	0.0	0.0
normal	0.0	0.0	0.0	0.0	0.0
ae su2 vs. normal					
ae su2	0.0	1.9	0.0	0.0	0.0
normal	2.9	1.5	0.0	0.0	0.0
ae wx vs. normal					
ae wx	2.1	0.0	0.0	0.0	0.0
normal	0.0	0.0	0.0	0.0	0.0

[a] Amylose-extender (*ae*), dull (*du*), sugary-2 (*su2*), waxy (*wx*).

TABLE V
Three-Class Principal Component Analysis (PCA) Calibrations for Discrimination of Double-Mutant Genotypes from Single-Mutant Counterparts Evaluated by % of Validation Samples Misclassified

Genotypic Class[a]	% Misclassified Using Multiple Factors					
	7	8	9	10	12	14
ae du vs. *ae* vs. *du*						
ae du	30.5	13.6	11.9	15.3	13.6	6.8
ae	23.8	23.8	18.1	18.1	24.8	17.1
du	15.4	3.8	0.0	0.0	0.0	23.1
du su2 vs. *du* vs. *su2*						
du su2	21.8	10.3	11.5	7.7	2.6	1.3
du	26.9	23.1	23.1	15.4	3.8	3.8
su2	44.0	38.0	42.0	44.0	22.0	6.0
du wx vs. *du* vs. *wx*						
du wx	4.0	2.0	4.0	6.0	0.0	0.0
du	0.0	3.8	0.0	3.8	0.0	0.0
wx	1.9	0.0	0.0	0.0	0.0	0.0
ae su2 vs. *ae* vs. *su2*						
ae su2	16.7	27.8	25.9	11.1	24.1	31.5
ae	10.5	9.5	9.5	17.1	9.5	3.8
su2	3.8	0.0	0.0	0.0	0.0	0.0
ae wx vs. *ae* vs. *wx*						
ae wx	12.8	6.4	2.1	2.1	0.0	2.1
ae	12.4	9.5	13.3	8.6	1.0	0.0
wx	1.9	0.0	0.0	0.0	0.0	0.0

[a] Amylose-extender (*ae*), dull (*du*), sugary-2 (*su2*), waxy (*wx*).

TABLE VI
Two-Class Partial Least Squares Analysis (PLS) Calibrations for Discrimination of Double-Mutant Genotypes from Single-Mutant Counterparts Evaluated by % of Validation Samples Misclassified

	% Misclassified Using Multiple Factors					
Genotypic Class[a]	7	8	9	10	12	14
ae vs. other *ae* double-mutant counterparts						
ae du vs. *ae*						
ae du	16.9	15.2	15.2	15.2	10.2	8.4
ae	16.8	10.3	8.4	11.2	4.6	3.7
ae su2 vs. *ae*						
ae su2	18.5	20.3	24.1	16.6	14.8	14.8
ae	20.5	23.4	23.4	20.6	16.8	15.8
ae wx vs. *ae*						
ae wx	4.2	6.4	2.1	2.1	2.1	0.0
ae	3.7	3.7	3.7	2.8	1.9	0.0
du vs. other *du* double-mutant counterparts						
du su2 vs. *du*						
du su2	0.0	0.0	1.3	1.3	1.3	1.3
du	3.8	0.0	0.0	0.0	0.0	0.0
ae du vs. *du*						
ae du	1.7	0.0	0.0	0.0	0.0	0.0
du	7.8	0.0	0.0	0.0	0.0	0.0
du wx vs. *du*						
du wx	2.0	0.0	0.0	0.0	0.0	0.0
du	0.0	0.0	0.0	0.0	0.0	0.0
su2 vs. other *su2* double-mutant counterparts						
ae su2 vs. *su2*						
ae su2	7.4	1.9	0.0	0.0	0.0	0.0
su2	10.0	0.0	0.0	0.0	0.0	0.0
du su2 vs. *su2*						
du su2	10.2	9.0	7.7	9.0	1.2	1.2
du	4.0	4.0	0.0	0.0	8.0	8.0

[a] Amylose-extender (*ae*), dull (*du*), sugary-2 (*su2*), waxy (*wx*).

TABLE VII
Two-Class Partial Least Squares Analysis (PLS) Calibrations for Discrimination of Low (46–57%) and High (59–76%) Amylose Grain Samples Within *ae* Genotype Evaluated by % Misclassification of Validation Set

			% Misclassified Using Multiple Factors			
Genotypic Class[a]	Calibration (*n*)	Validation (*n*)	10	12	14	16
ae (low) vs. *ae* (high)						
ae (low, 46–57%)	40	40	42.5	27.0	30.0	30.0
ae (high, 59–76%)	40	40	10.0	15.0	10.0	12.5

[a] Amylose-extender (*ae*).

SUMMARY

Classification models using NITS spectra could be useful for a limited number of mutant genotypic classes. These classes are predominantly those with unique starch structure or amylose content. Classification worsens when genotypic classes has overlapping levels of amylose. In addition, two-class models consistently outperformed three-class and multiclass models. Perhaps the most useful calibration would be a three-way model, which discriminated well among the genotypes normal, *ae,* and *wx,* which are the only classes currently produced in significant volumes.

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