

An Improved Method for Using a Microsatellite in the Rice Waxy Gene to Determine Amylose Class

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ABSTRACT

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Rice (*Oryza sativa* L.) breeders must evaluate progeny across multiple years and locations in part due to environmental effects on amylose content, the primary constituent that influences rice end-use quality. A microsatellite correlated with the various classes of apparent amylose content in rice has been used to decrease the development time for the U.S. cultivars Cadet and Jacinto by several years. The objective of this project was to develop a relatively inexpensive method for assaying this microsatellite that is suitable for screening large numbers of progeny and to evaluate this method by analyzing a diverse set of breeding lines and cultivars. Rapid multiple-kernel (brown and milled), single kernel, and leaf tissue alkali DNA extraction procedures were developed. Enhanced resolution of allele classes and separation speed was achieved by

electrophoresing polymerase chain reaction (PCR) amplification products encompassing the *waxy* microsatellite in a polyacrylamide and Spreadex gel matrix using a triple-wide mini electrophoresis unit. For germ plasm characterization, allele scoring accuracy and speed were improved by loading standards, consisting of three microsatellite classes in a single lane, several times across the gel. The microsatellite explained 88% of the variation in the apparent amylose content of 198 nonwaxy U.S. cultivars and breeding lines of diverse parentage, grown in four locations. The utility of this method was demonstrated by one technician analyzing a breeding population of 142 progeny in 1.5 days using relatively inexpensive laboratory equipment.

Amylose is the most important grain constituent that influences rice end-use quality and it is the major determinant used across the world to define rice market classes. Based on the amylose content of milled rice, germ plasm is commonly categorized into several amylose classes or quality types: waxy (0–2%), very low amylose (3–9%), low amylose (10–19%), intermediate amylose (20–24%), or high amylose (>24%) types. In the United States, the high amylose class is further divided into two subclasses, those quality types suitable for commercial thermal processing and those that are not.

Methods for determining milled rice apparent amylose (AA) content are relatively rapid and reproducible, but they have shortcomings when used for classifying breeding lines (Webb 1972). Gomez (1979) reported that cultivars may be classified into different amylose groups depending on where they are grown. Environmental effects are known to cause AA content to vary up to six percentage points for a given cultivar (Juliano and Pascual 1980). The portion of the environmental effects on AA content which result from temperature variation is cultivar dependent (Asaoka et al 1985; Paule 1977; Resurreccion et al 1977). Also, higher levels of amylose are reportedly controlled either through partial or complete dominance. Thus, heterozygotes cannot be identified using phenotypic measures of AA content (Bollich and Webb 1973; McKenzie and Rutger 1983; Pooni et al 1993). Because of these shortcomings, breeders evaluate the AA content of progeny across multiple years and locations.

Bligh et al (1995) reported a polymorphic microsatellite in the *waxy* gene (*wx*) which codes for granule bound starch synthase. This (CT)_n (cytosine and thymine) repeat explained a large portion of the variation of AA content in 89 U.S. nonwaxy cultivars (Ayres et al 1997) and has been used to decrease the development time of Cadet [plant variety protection number 9900110 (pending) USDA-AMS, Beltsville, MD] and Jacinto [plant variety protection number 9900109 (pending) USDA-AMS, Beltsville, MD] cultivars which have processing characteristics different from those of U.S. mar-

keting classes. Although this marker is suited for screening breeding populations, its adoption as a routine method has not been feasible because DNA extraction techniques for microsatellite analysis are quite lengthy and the gel scoring methods have low accuracy.

The objectives of this research were to 1) develop a relatively inexpensive method for analysis of the *wx* microsatellite that is suited for screening large numbers of progeny; and 2) test the method using a breeding population, advanced lines of diverse parentage, and cultivars.

MATERIALS AND METHODS

Method development was performed using leaves from two-week-old seedlings and brown and milled rice of cultivars Lemont, Caloro, Bengal, Rico1, CI 5309, Kaybonnet, Dixiebelle, Pokhareli Masino, and Te Tep. These cultivars were chosen because together they possess each of the nine microsatellite alleles previously reported by Ayres et al (1997) and Bergman et al (*in press*) (Fig. 1). The 200 entries in the 1999 Uniform Regional Rice Nursery (URRN) were also used to evaluate the method. These entries include advanced lines from the Texas, Louisiana, Arkansas, and Mississippi rice breeding programs and several cultivars. Rough rice samples were obtained from one field replicate of each entry grown in the four states. The samples were milled by each of the breeding programs using a McGill mill #2 (Houston, TX). Samples were also obtained from a population of 142 F₇ breeding lines developed from a cross between Dellmont cultivar and B8462T3-710 line as described by Ahn et al (1993) and grown during 1999 in Beaumont, TX. Dellmont and B8462T3-710 have conventional U.S. long grain and superior commercial thermal processing quality, respectively. Rough rice samples from one field replicate of each of the breeding progeny were milled using a brush mill (Precision Machines, Lincoln, NB). Standard cultural management practices were used in each state from which samples were obtained. Processed rice and extruded bran samples were provided by Riviana Foods (Houston, TX).

The following variables were studied during method development, NaOH molarity (0.4, 0.5, 1.0, and 1.5), sample (% w/v) in NaOH solution (5, 6.25, 7.5, and 25), need for vortexing and centrifugation, kernel storage age, extraction buffer pH (7.0, 7.5, and 8.0), and DNA extract age (16 hr, one, and two weeks). Also, gel electrophoresis conditions evaluated were separating gel, percent acrylamide (8, 10, and 12), Spreadex polymer native acrylamide-bis (NAB) concentration, need for stacking gel, and running time (1.75 and 2.75 hr).

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After optimization, the method used for DNA extraction from brown or milled rice kernels was 10 kernels incubated in 4 mL of 0.5M NaOH, overnight (16 hr), at room temperature. Individual kernels were extracted with 0.4 mL of NaOH. When extracting leaf tissue or processed food, 20 mg of material to 0.4 mL of NaOH was used. Tubes were vortexed briefly and then centrifuged at $825 \times g$ (model GR2022, Jouan). DNA extracts (20 μ L) were combined with 120 μ L of 100 mM Tris-HCl (pH 7.5) and 4 μ L of this solution was used in the polymerase chain reaction (PCR) assay. Each PCR reaction (20 μ L total volume) consisted of 2.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM deoxy-nucleotides, 0.2 μ M 484 oligo, 0.2 μ M 485 oligo (Operon Technologies, Alameda, CA), and 0.5 units of Taq polymerase (Life Technologies, Rockville, MA). Using a thermocycler (model 9700, Perkin Elmer, Norwalk CT), denaturation was at 95°C for 4 min, followed by 37 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 45 sec, and a final period at 72°C for 5 min.

The gel electrophoresis was performed using a triple-wide mini vertical gel electrophoresis system (C.B.S. Scientific, Del Mar, CA). The separating and stacking electrophoretic gels consisted of 12 and 4% acrylamide and bisacrylamide (29:1) (Midwest Scientific, Valley Park, MO), respectively, and 1X Spreadex Polymer NAB (Elchorn Scientific, Cham, Switzerland). Electrophoresis gels were run at 200 V for 2.75 hr and bands were stained for 10 min with GelStar

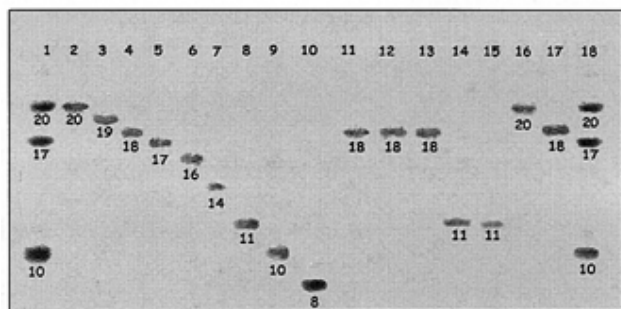


Fig. 1. Polymerase chain reaction (PCR) products from a microsatellite $(CT)_n$ (cytosine and thymine) associated with the *waxy* gene separated using polyacrylamide plus Spreadex gel electrophoresis. Numbers above bands indicate lane number while numbers underneath designate the bands microsatellite class. Lanes 1 and 18, controls used for scoring the gel, $(CT)_{20}$, $(CT)_{17}$ and $(CT)_{10}$; 2–10, one band for each known allele for the *waxy* microsatellite; 11–13, Bengal (leaf material, hulled kernels, and milled kernels); 14, Dixiebelle, milled parboiled kernels; 15, L205, stabilized bran; 16, Cypress, instant rice kernels; and 17, Bengal, crisp rice.

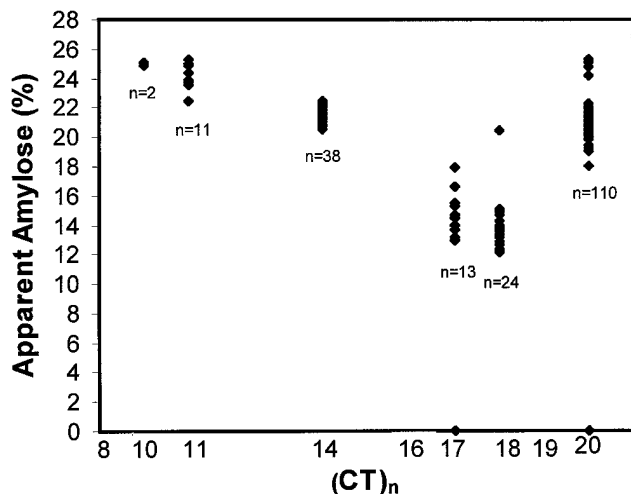


Fig. 2. Mean apparent amylose content and microsatellite $(CT)_n$ (cytosine and thymine) class of 200 entries in the 1999 U.S. Uniform Regional Rice Nursery grown in four states.

nucleic acid stain (Bio Whittaker Molecular Applications, Rockland, MA). Electrophoretic bands were scored using standards consisting of three microsatellite classes in a single lane loaded several times across the gel, and PCR product of each microsatellite allele loaded in consecutive lanes in the middle of the gel (Fig. 1). Bands were visualized using a blue-light transilluminator (Parker Reader, Clare Chemical, Denver, CO) and recorded with a Spot II camera (Diagnostic Instruments, Sterling Heights, MI). Multichannel pipettors and a 96-well microtiter plate format were used throughout the procedures to increase throughput.

Standards were produced by separating PCR products of samples run using the above protocol in a 3% electrophoretic agarose gel. Plugs were taken of each electrophoretic band, diluted in 200:1 Tris-EDTA buffer (10 mM Tris, pH 8.0, 0.5 mM EDTA), and amplified in large scale PCR assays. These PCR products were precipitated by adding 1:10 sodium acetate buffer (pH 5.2, 3 M) and 1:2.2 ethanol (100%), centrifuging, and storing at 0°C, for 20 min. The DNA pellet was washed with 1:1 ethanol (70%), dried and resuspended in 10 mM Tris-HCl buffer (pH 8.0, 0.5 mM EDTA) and PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4).

Apparent amylose was determined in duplicate using a colorimetric method and reported on an as-is basis (Webb 1972). Analysis of variance and means separation using the Student-Newman-Keuls test was performed with SAS v.7 (SAS Institute, Cary, SC).

RESULTS AND DISCUSSION

DNA Extraction Optimization

Milled rice samples of cultivars possessing each of the nine microsatellite alleles were successfully analyzed using the optimized DNA extraction procedure (Fig. 1). The alkali extraction method worked

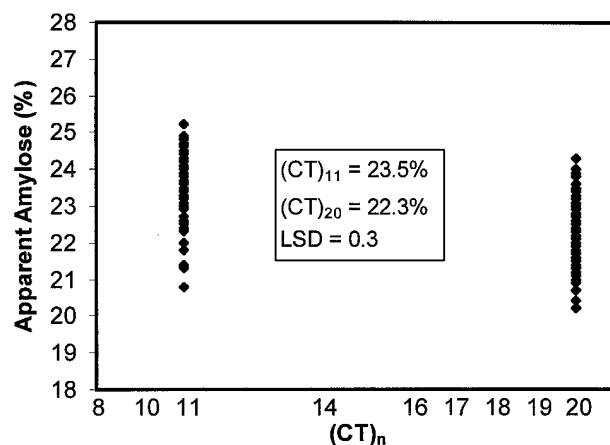


Fig. 3. Apparent amylose content and microsatellite $(CT)_n$ (cytosine and thymine) class of 142 progeny developed from a cross between Dellmont and B8462T3-710. Mean apparent amylose values for the progeny in each of the two $(CT)_n$ classes are listed in the legend. Least significant difference ($P < 0.05$).

TABLE I
Apparent Amylose Content of Six Microsatellite Classes $(CT)_n$ of 198 Entries of the 1999 U.S. Uniform Regional Rice Nursery^a

Microsatellite Class	Apparent Amylose (%) ^b
$(CT)_{10}$	25.00a
$(CT)_{11}$	24.08a
$(CT)_{14}$	21.59b
$(CT)_{20}$	21.33b
$(CT)_{17}$	14.72c
$(CT)_{18}$	13.82c

^a Apparent amylose content for each entry is the mean of samples obtained from plots grown in Texas, Mississippi, Louisiana, and Arkansas. Number of entries included in each microsatellite class is reported in Fig. 2.

^b Values followed by the same letter are not significantly different ($P < 0.05$).

equally well for the nine cultivars using leaf tissue and kernels that were dehulled or milled (data not shown). As an example, results from leaf tissue, dehulled kernels, and milled kernels of the cultivar Bengal are shown in Fig. 1. Approximately 2% of the rice samples analyzed using this extraction technique in this and subsequent tests were initially recalcitrant to amplification. However, in instances when a 10 kernel sample DNA extract did not amplify, results were generally obtained by analyzing 10 kernels individually.

Many DNA extraction protocols and kits suitable for use with plant material have been reported. Zimmermann et al (1998) performed a quantitative and qualitative evaluation of nine DNA extraction methods using various soy bean-based products. They determined that resin-based DNA extraction methods result in relatively low amounts of high quality DNA. Also, they and Steiner et al (1995) determined that DNA obtained from simpler and faster protocols using alkali achieved greater amounts of poorer quality DNA that did not reliably produce PCR products. Although the extraction method reported here likely produces DNA of poor quality, the method's speed advantage clearly outweighs the small portion of samples that present amplification difficulty.

Mixtures of two cultivars with different microsatellite alleles were examined using the bulk kernel extraction procedure. Mixtures could be identified in ratios of 1:1 to 7:3 kernels, but not in ratios of 8:2 or 9:1 (data not shown). When multiple bands were found for bulked samples, the PCR assay was run using several single kernels. Single kernel analysis revealed whether the bulk sample was a mixture of more than one genotype or due to heterozygous individuals. PCR products were obtained using brown rice kernels from a sample of Rexoro that had been stored for 59 years, at 0°C; thus, kernel storage age does not appear to affect this DNA extraction procedure. This method was also successful in determining the (CT)_n allele of rice used in food products such as parboiled rice, extruded bran, instant rice, and crisped rice (Fig. 1). DNA extracts stored for up to two weeks under refrigeration did amplify. However, PCR products were obtained from a greater percentage of samples when the DNA extracts were used in the PCR assay immediately after centrifugation.

Taylor et al (1993) has described a suitable DNA extraction procedure as one that works with targeted tissue, is relatively simple and reliable, and where DNA of sufficient purity is obtained. The DNA extraction method reported here meets these requirements. Other advantages that make the DNA extraction procedure suitable for high throughput screening include its low cost and use of nonorganic solvents.

Electrophoresis Optimization

During initial method development, PCR products were separated using an electrophoretic sequencing gel instrument (Life Technologies model S2001, Rockville, MD). This method was effective in increasing the number of samples that could be separated at one time. However, it was extremely difficult to score the electrophoretic bands because the PCR products traveled at different rates depending on where they were loaded across the electrophoretic gel. Loading standards across the gel helped with scoring accuracy. Greater throughput and ease of scoring was achieved, however, by using a triple-wide electrophoretic mini-gel system. Polyacrylamide plus Spreadex polymer NAB was necessary for adequate band separation using the mini-gel system. The method time was also shortened by staining the electrophoretic gels with GelStar nucleic acid stain rather than the silver stain technique used by Ayres et al (1997).

Microsatellite Association with Amylose Class and Use as a Breeding Tool

The improved speed of the procedure for analyzing the *wx* microsatellite reported here can be appreciated by comparing it with the previous methods of determination of the microsatellite and wet chemistry apparent amylose. Using the method reported here, one

technician can analyze ≈575 samples per week, compared to ≈150 samples per week using the method by Bligh and Jones (1995) or Ayres et al (1997). Instrumentation is currently available that could further increase the efficiency of the microsatellite assay but it may be cost-prohibitive for some breeding programs. The AA content of ≈400 milled rice samples can be analyzed by one technician in a week using the method by Webb (1972). Thus, this method is more efficient at determining the microsatellite and predicting AA class.

All of the *wx* marker alleles reported by Ayres et al (1997) were found in the URRN entries except the (CT)₁₉, (CT)₁₆, and (CT)₈ (Fig. 2). The (CT)₁₀ allele reported by Bergman et al (*in press*) was also identified. Nonwaxy entries with a (CT)₁₀ or (CT)₁₁ allele, a (CT)₁₄ or (CT)₂₀ allele, and a (CT)₁₇ or (CT)₁₈ were categorized as high amylose, intermediate amylose, and low amylose types, respectively, according to the wet chemistry AA method (Table I). One of the two waxy entries had a (CT)₁₇ allele and the other had a (CT)₂₀ allele.

The U.S. rice germ plasm base is very narrow and thus it was not surprising that the URRN entries did not possess all of the known *wx* marker alleles (Dilday 1990). Also, previously reported cultivars with a (CT)₁₉ allele were California medium grain types, we studied Southern U.S. genotypes (Ayres et al 1997). No U.S. cultivars have been reported that have the (CT)₁₆ or (CT)₈ allele (Ayres et al 1997). Work by Ayres et al (1997) found that nonwaxy U.S. germ plasm with a (CT)₁₈ allele were low amylose types and one accession with the (CT)₈ allele was an intermediate type. Examining germ plasm from 53 countries, Bergman et al (*in press*) reported that nonwaxy accessions with the (CT)₈ microsatellite allele were high amylose types, whereas accessions with a (CT)₁₈ ranged from low to intermediate amylose types. Samples in both of these studies were obtained from different growing environments. Further work is needed to clarify the genotype by environment interaction using diverse germ plasm grown in the same environment. Until these issues are better understood, breeders should have good estimates of the AA contents and (CT)_n classes of their parental breeding lines before using the *wx* microsatellite to make selections in segregating populations.

The variation in AA content for the nonwaxy entries in the URRN explained by the microsatellite was 88%. Ayres et al (1997) reported that 82.9% of the variation was explained for the AA content of 89 cultivars, primarily of U.S. origin. Our results indicate that a strong relationship between AA content and the microsatellite associated with the *wx* gene holds across the divergent genetic backgrounds currently in U.S. breeding programs and across environments.

The breeding population examined is segregating for superior commercial thermal processing and conventional U.S. long grain quality (Fig. 3). The data for the URRN entries and Ayres et al (1997) show that in all cases the (CT)₁₁ allele is associated with superior processing quality types, while the (CT)₂₀ allele is found in conventional U.S. long grain types. Rice with superior thermal processing quality are also high amylose types that have greater kernel integrity after thermal processing, while conventional U.S. long grains are in the intermediate amylose class. Although these two rice quality types can usually be differentiated using mean AA content across several environments, the AA content for individual lines grown in one environment often fails to distinguish between the two quality types. Consequently, progeny with superior processing quality are generally identified relatively late in the breeding process by examining their mean AA across multiple environments and by determining pasting properties, a relatively time-consuming procedure (AACC 2000).

Figure 3 demonstrates the potential utility of using the microsatellite to categorize breeding lines in different amylose classes that are a challenge to distinguish between using the wet chemistry AA method, due to environmental effects. The microsatellite alleles of Dellmont and B8462T3-710 are (CT)₂₀ and (CT)₁₁, respectively (data not shown). The Dellmont and B8462T3-710 progeny with a (CT)₂₀ allele had a mean of 22.3% AA, while those in the (CT)₁₁

class had a higher mean of 23.5% AA (Fig. 3). Thus, although the mean AA values for the two quality types were significantly different, many of the lines had an AA content that would cause it to be misclassified. It would be very difficult for a breeder to select progeny accurately in this population with the desired quality using only the AA data. Without the use of the microsatellite, selecting for the superior processing types in this population would require that it be grown in several additional environments, and AA content and pasting properties be determined.

Several heterozygotes were identified in this population using the *wx* marker. Wet chemistry measurements of AA content would not have provided breeders this valuable information. Our results indicate that the microsatellite assay is more effective at classifying segregating progeny for amylose class than traditional methods that are sensitive to the production environment.

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