Pre-breeding for waxy proso millet by phenotyping and marker-assisted selection

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ABSTRACT

Proso millet (Panicum miliaceum L.) is important in some Kazakhstan food, this research objective was to develop pre-breeding waxy proso millet resource for breeding new varieties by using phenotyping and marker-assisted selection. The amylose content of the endosperm starch in a collection of 18 proso millet cultivars widely used in northern Kazakhstan were evaluated and ranged from 14.6% to 34.8%. The introduced glutinous millet accessions of PI436626 (Lung Shu 18), PI436625 (Lung Shu 16) were selected to cross with local cultivars (Saratovskoe 6 and Pamyati Bersieva). The potassium iodide (KI) staining method and PCR analysis using the 9bF/15delRB primer were used to identify the waxy trait for selection of the F2-F3 generation. Seeds staining dark blue to black were scored as wild type, while waxy seeds were stained as pinkish or amber to reddish-brown color. The hybridological analysis of inheritance of the “waxy” trait suggested that the segregation ratios for waxy endosperm in the F2 and F3 populations was close to 15:1 of the caryopses with blue and brown colors. PCR analysis with the 9bF/15delRB primer confirmed that this specific primer flanks a 123 bp fragment in amylose samples, while in glutinous samples, this primer promotes the amplification of 108 bp long PCR product (15 nucleotides deletion). Finally, the waxy lines were obtained and could be used for further testing.

Key words: Amylose content, glutinous variety, hybridization, Panicum miliaceum, proso millet, waxy gene.

INTRODUCTION

Common millet (Panicum miliaceum L.) is the oldest valuable cereal and forage crop in the world. Its cultivation began 10000 years ago in Northern China (Lu et al., 2009). Proso millet is a short day C₄ plant and is one of the most valuable crops for universal use. The green pulp has a quality that can compete with the green pulp of maize, sorghum, and Sudan grass. Millet can rightfully be considered the main crop culture of ancient Europeans (Sidorenko et al., 2012). The appearance of millet in Canada dates back to the 17th century (Baltensperger et al., 2002). In the modern world, millet is cultivated in Asia, Australia, North America, Europe, and Africa (Brink and Belay, 2006; Zhang et al., 2019). Asian countries use millet as a food crop; USA is actively engaged in its growing for livestock and poultry (Dikshit and Sivaraj, 2013; Santra and Rose, 2013).

World production of millet is at a fairly high level and occupies a significant place. According to FAO, the sown area of millet ranks sixth in terms of sown areas (34.7 million hectares) and gross grain harvest (31.6 million tons) among cereals, second only to wheat, rice, barley, corn, and sorghum. Millet is cultivated in 30 countries of the world, including 18 European countries. The main grain producers of this crop are Russia, India, USA, China and Ukraine (Zotikov et al., 2012). Due to its early maturity, drought resistance, productivity, and other valuable biological and economic characteristics, this crop can be widely used in production (Minina, 2014).
In Kazakhstan, proso millet is one of major cereal crops distinguished by its high drought and salt tolerance as well as low response to sowing dates, which is especially important in the dry steppe zone (Tsygankov et al., 2004). In breeding of millet, cereal and forage directions are distinguished, as well as creation of special-purpose varieties, in particular, varieties with the starch consisting entirely of amylopectin. According to the qualitative composition of starch, cereals, including millet, are subdivided into waxy type, the starch of which is almost 100% amylopectin, and common (wild) millet, in which up to 30% of starch is amylose (Sakamoto, 1987; Hunt et al., 2013). In high-amylose samples of millet, the amylose content ranges from 20% to 32% (Beleia et al., 1980; Kim et al., 2012), samples with an amylose content of 3.3%-11.4% belong to low-amylose varieties (Hoover et al., 1996; Kim et al., 2009).

It has been established that the common type of starch in proso millet is controlled by the dominant alleles of \( W_x \) gene, which encodes in cereals the synthesis of the enzyme associated with granule-bound starch synthase (GBSS), whereas the waxy type (amylopectin) by its recessive \( wx \) alleles (Fukunaga et al., 2002; Araki et al., 2012). Since millet is a tetraploid plant, the genotype of different varieties may contain one \( W_x \) gene or two (\( W_x1 \) and \( W_x2 \)). If the endosperm of common millet varieties contains both types of starch molecules, it can be concluded that the dominant alleles of the \( W_x \) gene in such forms function only at a certain stage. Hunt et al. (2010) showed the presence of two different loci of these genes designated “L type” and “S type”.

Waxy (glutinous) forms of proso millet (endosperm starch in which lacks amylose) have been known since the 19th century (Hixon and Brimhall, 1968). Waxy proso millet obviously occurs widespread across Asia (Kimata and Negishi, 2002). A significant part of millet varieties cultivated in USA and European countries possesses the “wild” type of starch, presenting a mixture of amylose and amylopectin, while in Asian countries breeding programs aimed at creating varieties of the waxy type, in which content of amylose is insignificant (Graybosch and Baltensperger, 2009).

On the market today waxy types of proso millet are in high demand due to their increased stickiness and food value. Millet obtained from glutinous forms of millet is a valuable dietary product, which removes toxic compounds, toxins, and even heavy metal ions from the body. It should also be noted that millet is capable of removing antibiotics from the body (Kim et al., 2012).

The State Register of Breeding Achievements recommendation for use on the territory of the Republic of Kazakhstan includes 21 varieties of proso millet, there are no glutinous varieties among them. The price of glutinous millet on American and Asian markets is estimated at around 4 USD kg\(^{-1}\), while on the local market the price of ordinary millet is only 1 USD kg\(^{-1}\). The purposes of the present investigation were to identify waxy types of proso millet from the Kazakhstan collection based on biochemical and molecular analysis; evaluate the inheritance of the waxy trait after crossing with amylose (wild) type proso millet; and create pre-breeding sources of waxy proso millet for breeding new Kazakhstani varieties.

**MATERIALS AND METHODS**

**Breeding material**
Varieties and samples of proso millet (*Panicum miliaceum* L.) from local and world collection of various ecological and geographical origins were used as a starting material. The 18 Kazakhstan proso millet cultivars were bred in the last 40 yr. The low-amylose millet accessions of PI346946, PI436622 (Lung Shu 5), PI436623 (Lung Shu 7), PI436624 (Lung Shu 14), PI436625 (Lung Shu 16) and PI436626 (Lung Shu 18) were used as the waxy donors, which were obtained from the North Central Regional Plant Introduction Station (NCRPIS), Iowa State University, Ames, Iowa, USA.

The work was carried out in laboratory and field conditions. Laboratory studies were carried out at the Scientific Research Platform of Agricultural Biotechnology (RPAB) at the Saken Seifullin Kazakh Agrotechnical University, Nur-Sultan, Republic of Kazakhstan. Field experiments were carried out from May to September in the 2019 and 2020 growing seasons in the breeding nursery of the Scientific Production Center of Grain Farming named A.I. Baraev (Shortandy village-1, Shortandy district, Akmola region, Republic of Kazakhstan) in the dry steppe zone of the Akmola region.

**Determination of total amylose content in millet grain by \( I_2\)-KI**
Grain starch amylose concentrations were determined according to Singh and Adedeji (2016). Millet samples containing 100.0 ± 0.1 mg DM were weighed and mixed with 1 mL 95% ethanol and 9 mL 1 N solution of NaOH, transferred to
100 mL volumetric flasks. After being kept at room temperature for 10 min, flasks were heated on a water bath at 100 °C for 10 min, and left to cool at room temperature. The resulting mixture was diluted to 100 mL with dH2O and stirred. An aliquot of the starch solution (5 mL) was transferred to a 100 mL volumetric flask containing 50 mL dH2O. Then, 1 mL 1 N solution of acetic acid and 2 mL 0.2% iodine solution (I2-KI) were added to the same flask; it was filled to a volume of 100 mL and left for 20 min at room temperature. Dark blue to black were scored as wild type, while waxy type was noted by their pinkish or amber to reddish-brown color. The optical density was measured on a spectrophotometer (PE-5400UV, Erkos, Moscow, Russia) at a wavelength of 620 nm. The experiment was carried out in triplicate, results were processed statistically using the Microsoft Excel program.

**Genomic DNA extraction from millet seedlings**

For molecular genetic analysis, DNA was isolated from 7-d-old chlorophyll-free seedlings by the modified cetyl trimethylammonium bromide (CTAB) method (Murray and Thompson, 1980) as following: 100-200 mg seedlings were placed in a 2 mL test tube, where 400 μL CTAB 2% extraction buffer was added, and gently ground with a plastic rod; 10 μL RNase was subsequently added to the tube, with the latter incubated for 60 min at 65 °C in a water bath, periodically shaken; 400 mL chloroform-isoamyl alcohol were added and centrifuged for 1 min at maximum speed (13 000 rpm). The upper phase was carefully removed with a pipette, transferred to a new tube and 350 mL cold isopropanol was added and mixed thoroughly. It was centrifuged for 5 min at maximum speed (13 000 rpm), the alcohol was discarded, and the DNA was left in an open tube for drying. Dried DNA was dissolved in distilled water, the concentration was determined using UV-Vis spectrophotometers (NanoDrop 2000, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

**PCR analysis**

To identify the Wx gene, PCR was performed using the 9bF/15delRB primer, which amplified the region of Intron 9/Exon10, and detected the 15 bp deletion in wax type (Araki et al., 2012). The sequence (5’–3’) of 9bF/15delRB marker was as 9bF: CAAGGAAGCATTTCAGGCCATCGCT, and 15delRB: TGCTCCTCCAGCCTGCCGACA, which were synthesized by Applied Biosystems (Foster City, California, USA).

The PCR reaction mixture of 15 μL contained: 8 μL NZYTaq II 2 × Green Master Mix (NZYTech, Lisboa, Portugal), 5.2 μL ddH2O, 1 μL 10 μM each primer (F, R) and 100-150 ng DNA template. The amplification was carried out in a thermal cycler (SimpliAmp, Thermo Fisher Scientific) under the following conditions: 4 min at 94 °C; 35 cycles with 94 °C 1 min, 59 °C 1 min, 72 °C 1 min; further at 72 °C 8 min. Products of PCR were separated using 1.5% agarose gel electrophoresis (molecular biology grade, Invitrogen, Waltham, Massachusetts, USA) in Tris-acetate-EDTA (TAE; Tris-acetate 40 mM, EDTA 1 mM, pH 8.0), and 100 bp ladder (BioLabs, London, UK) was used as a marker of molecular weight. Electrophoresis was performed at a constant voltage of 120 V for 1 h. After electrophoresis, the results of amplification were visualized using a gel documenting system (TCP-20.MC, Vilber Lourmat, Eberhardzell, Germany, 2010).

**Crosses and selection of low-amylose and waxy proso millet**

To obtain low-amylose pre-breeding materials, the world standards of glutinous accessions PI436626 and PI346946 were used as recipient forms, to cross with some Kazakhstan cultivars, as the pollen donors. For hybridization, the parents were sown in vegetation vessels for three periods with an interval of 10 d to synchronize their flowering periods. Crosses were made at the beginning of the flowering stage, when the upper and middle parts of the panicle were blooming. Manual castration was carried out, 20-30 of the most developed spikelets were left on one panicle, the flower films were carefully opened, and anthers were removed with tweezers. A parchment insulator was put on the panicle. Pollination was carried out the next day by opening the flower and applying the paternal pollen to the stigma of the pistil. The combination scheme, date and time of pollination, number of plot and row number were stamped on the isolator. Upon full ripeness, the percentage of seed set was calculated by the number of formed grains and seeds were harvested.

Resulting hybrids and parents were manually planted in the field in a hybrid nursery, the area of the plot was 1 m², the arrangement of the plots was systematic. The F1 seeds were then harvested, and the F2 populations were planted next year, seeds of each individual were harvested separately, then analyzed by potassium iodide (KI) staining method. The KI staining method was used to identify grains of waxy type millet, as the endosperm of glutinous types with the recessive
The *wx* gene was colored brown, while that of the amylose types with the dominant *Wx* gene was colored blue. The phenotypic waxy endosperm is dense in consistency, but matte in color with a high content of dextrins, which impair the technological and culinary qualities of cereals (Dzyuba et al., 2015).

**RESULTS**

**Identification of the waxy gene in the millet varieties in Kazakhstan**

In creating glutinous forms, the content of amylose in the grains of millet varieties from Kazakhstan in the last 40 yr was estimated by biochemical screening (Table 1).

The results showed that there were great variations in amylose content among the examined varieties cultivated in Kazakhstan, which ranged in 14.6%~29.3%. According to the classification, these varieties belong to the medium and high amylose groups, which suggested the absence of waxy samples of millet in Kazakhstani breeding.

As waxy proso millet has not been previously bred in Kazakhstan, there is no information on *waxy* genes of Kazakhstan varieties. The PCR identification of the allelic state of the *waxy* gene using the 9bF/15delRB marker showed that all the varieties in Kazakhstan were with the Wax loci, while the glutinous standards PI346946 from the USDA and MaZhaYan from Chinese collection were with the wax loci. PCR with marker 9bF/15delRB clearly amplified the 123 bp product typical for medium and high amylose varieties, as shown for the Kazakhstan varieties, while the 108 bp product for the glutinous variety. In this case, glutinous samples differ from amylose by 15 bp deletion in the S region of the *Waxy* gene locus.

**Segregation of waxy traits in the F2-F3 progeny**

Three crosses were carried out as PI436626×Saratovskoe 6 (P1), PI346946 with Pamyati Bersieva and Kokshetavskoe 66 (P2 and P3, respectively), the setting of hybrid seeds during manual castration ranged from 5.0% to 47.2%. The resulting hybrid caryopses were sown in the field to produce the F1 generations. After seed maturation, each hybrid combination was harvested separately and F1 sterility was determined. In the field, a hybrid nursery of F1-F2 hybrids and their parental forms was established to select the promising low-amylose forms of millet from splitting F2-F3 populations. Each plant in F1-F2 generation was visually analyzed (Figure 1).

**Table 1. Amylose content of proso millet varieties from Kazakhstan.**

| Variety          | Year of admission | Origin | Vegetation period | Amylose % |
|------------------|-------------------|--------|-------------------|-----------|
| Saratovskoe 3    | 1981              | Russia | 79                | 25.7      |
| Uralskoe 109     | 1981              | Kazakhstan | 89          | 28.9      |
| Saratovskoe 6    | 1985              | Russia | 79                | 28.8      |
| Shortandinskoe 7 | 1994              | Kazakhstan | 82          | 28.4      |
| Omskoe 11        | 1994              | Russia | 79                | 24.8      |
| Saratovskoe10    | 2006              | Russia | 80                | 26.5      |
| Pamyati Bersieva | 2009              | Kazakhstan | 75        | 23.4      |
| Shortandinskoe10 | 2009              | Kazakhstan | 82        | 29.3      |
| Yarkoe 3         | 2009              | Kazakhstan | 83        | 25.5      |
| Shortandinskoe11 | 2011              | Kazakhstan | 82        | 28.7      |
| Pavlodarskoe     | 2011              | Kazakhstan | 82        | 29.4      |
| Yarkoe 5         | 2012              | Kazakhstan | 82        | 26.9      |
| Barnaulskoe 98   | 2013              | Kazakhstan | 84        | 28.8      |
| Yarkoe 7         | 2015              | Kazakhstan | 82        | 28.6      |
| Yarkoe 6         | 2016              | Kazakhstan | 82        | 28.7      |
| Yarkoe 120       | 2017              | Kazakhstan | 82        | 28.1      |
| Pavlodarskoe 4   | 2017              | Kazakhstan | 82        | 20.1      |
| Yarkoe ubileinoe | 2019              | Kazakhstan | 82        | 24.8      |
Figure 1. Husk shape and color of obtained *Panicum miliaceum* F₁ hybrids.

Figure 1 clearly show that seeds of F₁ hybrids derived from PI436626×Saratovskoe 6 and PI346946×Pamyati Bersieva have the similar caryopsis color as the female parent, which suggested that color of the flower scales was inherited from the maternal form. These combinations were planted next year in the field conditions, and the F₂ populations were harvested separately. To identify a waxy trait for selection by using 2% KI solution, a hybridological analysis was performed on the F₂-F₃ generation of the crosses of PI436626×Saratovskoe 6 and PI346946×Pamyati Bersieva. Seeds staining dark blue to black were scored as wild type, while waxy seeds were stained as pinkish or amber to reddish-brown color (Figure 2).

In F₁ generation, all obtained hybrid caryopses were blue when stained with 2% KI, as domination of amylose endosperm, while the waxy parents were pinkish or amber to reddish-brown. Staining analysis in populations F₂-F₃ showed that the color of the endosperm was determined by their genotype, i.e., endosperm with a dominant *Wx* gene was colored blue, while endosperm with two recessive *waxy* genes was stained brown. Further we observed that the ratio of caryopses with blue and brown colors was close to 15:1, with χ² in the range of 0.06-0.07 at a probability between 0.90 < P < 0.75 (Table 2), which suggested that this trait was controlled by two dominant *Waxy* genes, and the staining the caryopses with 2% KI solution can be a better way for assist the selection of wax proso millet in the breeding program.

Figure 2. Starch granules of *Panicum miliaceum* stained with 2% potassium iodide solution: wild type stained dark blue (a), and waxy type stained reddish-brown (b).

| Crossing combinations   | Endosperm coloring | Number of tested grains | χ² | P       |
|-------------------------|--------------------|-------------------------|----|---------|
|                         |                    | Total | Actual | Theoretical |        |         |
| F₂ PI436626/Saratovskoe 6 | Blue/Brown         | 589   | 547.0/42.0 | 549.7/39.3 | 0.07   | 0.90 < P < 0.75 |
| F₂ PI346946/Pamyati Bersieva | Blue/Brown       | 676   | 631.0/45.0 | 630.9/45.1 | 0.07   | 0.90 < P < 0.75 |
| F₃ PI346946/Pamyati Bersieva | Blue/Brown       | 1983  | 1855/128  | 1859/124   | 0.06   | 0.90 < P < 0.75 |

Table 2. Hybridological analysis of F₂-F₃ generations of hybrids by endosperm color.
Evaluation of hybrids by biochemical and molecular markers

The amylose content in grain in F2-F3 individuals of PI346946/Pamyati Bersieva was estimated using biochemical analysis. And the results showed that amylose content in F2 hybrid populations ranged from 15% to 24%, while in F3 hybrids from 11% to 22% (Figure 3), it was observed a decrease in the content of amylose with self-pollination.

From F2 generation, individuals K2-M10 and K2-M19 were distinguished with a relatively low content of amylose, namely 11.6% and 13.5%. After analysis for amylose content, each panicle was analyzed visually according to their caryopsis shape as round character from the paternal shape and elongated character from the maternal shape. In spring, in the breeding nursery, the F3-F4 individuals from the population PI346946/Pamyati Bersieva were sown based on their shape in a separate plot, and their amylose content was estimated again after harvesting (Figure 4).

Among the studied samples, a sample was distinguished with a very low amylose content K1M4 with an elongated grain shape, as well as samples K1M3 and K1M1 with a round grain shape.

Subsequently, individuals of F3-F4 generation from PI346946/Pamyati Bersieva were analyzed by PCR using the 9bF/15delRB marker to identify the wx allele (Figure 5).

PCR analysis revealed that the most F3-F4 individuals were amylose type, three individuals of K1M1 (round), K1M3 (round) and K1M4 (elongated) with a product size of 108 bp, the glutinous forms of millet were identified. Thus, selected promising pre-breeding materials will be used for breeding Kazakhstan waxy proso millet varieties.

Figure 3. Amylose content of the Panicum miliaceum F3 populations of PI346946/Pamyati Bersieva.

Figure 4. Amylose content in Panicum miliaceum hybrid populations of the combination PI346946/Pamyati Bersieva F4 generations.
Amylose content is the most important biochemical indicator of grain quality. In high-amylose proso millet, the amylose content ranges from 20% to 32% (Beleia et al., 1980; Kim et al., 2012), while in low-amylose millet, amylose content is 3.3%-11.4% (Hoover et al., 1996; Kim et al., 2009). In the study carried out to create pre-breeding resources of waxy proso millet for breeding new Kazakhstan varieties, we focused on the assessment of amylose content in the grains of the initial breeding material. Among our materials, all varieties cultivated in Kazakhstan had an apparent intermediate and high-amylose content, and ranged from 14.6% to ~29.3%.

The amylose content in the waxy endosperm of millet is up to 3.5% and is controlled by the recessive alleles \( wx-1 \)/\( wx-2 \), and in the non-waxy endosperm by the dominant alleles \( Wx-1 \) and \( Wx-2 \) (Graybosch and Baltensperger, 2009). So far, the \textit{waxy} gene of proso millet varieties cultivated in Kazakhstan has been never studied using marker-assisted selection. PCR analysis with 9bF/15delRB marker for identification of \textit{Waxy} alleles confirmed that all Kazakhstan varieties included in the State Register are amylose type, which justifies the necessity of its breeding for introducing the \( wx \) loci.

Studies by a number of local scientists have shown that the content of amylose is a trait of polygenic nature and is stabilized only in F6-F7 generations in rice. When analyzing the samples obtained from PI346946/Pamyati Bersieva, it was found that the amylose content of F2 generations varies within 15%–24%, in F3 generations 11%–22%, in F4 generations 5.9%–19.0% (Figure 6), which suggest that there was a tendency towards a decrease in amylose along with the generation after selection.

Regarding the breeding strategy, to avoid the genetic unification of the assortment genetic sources previously uninvolved in crossing are required for hybridization work on creation of waxy forms of millet. Glutinous samples of proso millet from the world collection PI346946, PI436626 (‘Lung Shu 18’), PI436625 (‘Lung Shu 16’) are a valuable genetic source for creation of the first national glutinous millet variety with a complex of economically valuable traits by transferring the \textit{waxy} gene to local varieties. Involvement of these genotypes in breeding research will further contribute not only to a decrease in the content of amylose in grain, but also to an increase in the yield and adaptive potential of millet in Kazakhstan due to the expansion of the genetic basis of the crop.
CONCLUSIONS

Biochemical screening for quantifying the amylose content in the grain of millet samples and marker analysis for choosing the right parents are intended to create glutinous millet variety with a complex of economically valuable traits. The glutinous samples introduced were crossed with Kazakhstan varieties, assisted by phenotyping by staining and genotyping by marker analysis, low-amylose pre-breeding materials were obtained.

In this work, the KI staining method was used to determine the grains of waxy type proso millet. A dominant Wx gene produced grain with endosperm starch that stained blue-black with iodine, while a recessive wx gene produced grain with starch granules that stained red-brown. This method will be useful in phenotyping of much more germplasms to identify waxy type proso millet. Staining the endosperm on the F2-F3 generation with 2% KI solution showed that stained caryopses ratio with blue and brown colors was close to 15:1; suggesting that two genes control wax traits. As a result of the PCR analysis, polymorphism with 15-bp deletion was observed in three individuals of K1M1 (round), K1M3 (round) and K1M4 (elongated) hybrids in the region of Intron 9/Exon10 in the S gene.

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