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Identification of waxy genotype in sorghum genetic resources using waxy gene-based markers and iodine staining methods

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Abstract

Sorghum is widely recognized as a versatile crop with significant value in the production of food and energy. Sorghum with waxy trait (low amylose) is more palatable to the food industries and more appealing to consumers than nonwaxy variety. However, waxy sorghum has not yet been introduced to Indonesia, making it difficult to use sorghum to support the government of Indonesia's initiative to diversify the country's main foods. This study aimed at identify the waxy traits in a set of sorghum germplasm collected in Indonesia using waxy gene-based markers and iodine staining screening approaches. A total of 48 (27 introduced and 21 local) sorghum accessions collected in the IAARD gene bank along with 4 national sorghum varieties, which were subjected to both molecular analysis and iodine staining assay. The presence of waxy allele in the sorghum gene bank collection was identified using DNA markers associated with two waxy alleles (wx^a and wx^c). Iodine staining assay performed in the present study was applied to verify the waxy phenotype in sorghum grain and waxy genotypes in sorghum pollen. Based on molecular analysis and pollen staining, five local sorghum accessions were classified as heterowaxy sorghum. These accessions showed reddish-brown color in their endosperm after treated by iodine staining which confirmed them as waxy phenotype. These findings would be beneficial for further improvement of grain quality in sorghum breeding programs.

Keywords: allele; endosperm; grain; heterozygous; pollen.

Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is widely known as a multipurpose crop that is very important in food and energy production (Suarni, 2017). Sorghum has been named as a cereal to support food diversification programs in Indonesia, but there have been many constraint in the utilization and development of sorghum as an alternative source of carbohydrate (Widowati, 2010). Information on nutritional value, functional components, and physicochemical properties can greatly increase consumer's interests as well as accelerate public acceptance of sorghum as common food consumption (Trikoesoemaningtyas et al., 2015; Suarni, 2017).

Sorghum grains contain 73% carbohydrates, 10.4% protein, and 3.1% fat as macronutrient (Widowati, 2010). The protein content observed in sorghum is higher in rice and maize, making sorghum a favorable crop with high nutritional value (Widowati, 2010; Aderibigbe et al., 2022). In addition, sorghum has beneficial minerals such as iron (Fe) (3.82 mg 100 g^{-1}) that is beneficial to the human body's metabolism (Badigannavar et al., 2016). Sorghum also contains phenolic, flavonoid, and carotenoid compounds that have antioxidant activity (Mawouma et al., 2022). It also reported that phenolic compunds extracted from sorghum have antidiabetic effects (Chung et al., 2011).

The texture of cooked sorghum is one of the physicochemical properties influencing the quality of sorghum processing for rice-like foods. According to Widowati et al. (2010) and Rasyid et al. (2016), the texture is one of the food quality parameters that determine the level of consumer's acceptance of a food product. The amylose content in sorghum grain is reported to have a great impact on the texture of

cooked sorghum (Budijanto and Yuliyanti, 2012; Rasyid et al., 2016). Higher amylose content is associated with the viscoelasticity of cooked sorghum resulting in a harder texture in cooked sorghum. A low amylose content result in a softer and sticky texture of cooked rice sorghum (Luna et al., 2015).

Low amylose content in endosperm of sorghum grain has been recognized as waxy type. Sattler et al. (2009) reported that waxy sorghum type originates from mutations in the *granule-bound starch synthase I (GBSS I*) gene, which synthesize amylose. Consequently, this mutations dramatically reduce the activity of *GBSS I* which leads to a decrease in amylose content. The waxy phenotype in sorghum is regulated by a single gene in recessive form symbolized by *wx* (Jampala et al., 2012). It not only affects physicochemical properties such as texture and waxy phenotypes but also alter starch digestibility (Pedersen et al., 2007). Waxy sorghum has a higher digestibility of starch and protein than wild-type sorghum (Wong et al., 2009). Waxy sorghum is reportedly contain 41.15% of polyunsaturated fat which is needed in the human diet and can reduce the risk of atherosclerosis (Pontieri et al., 2020). These advantages can offer Indonesian nation's food diversification and sustainability program.

Several national sorghum varieties in Indonesia such as Numbu, Kawali, Bioguma, and Pahat were reported to have medium to high of amylose content (Budijanto and Yuliyanti, 2012; Suarni, 2017; Avif and Oktaviana, 2021). Currently, there is no Indonesian sorghum variety that has been reported to have a low amylose content in Indonesia. Therefore, it is necessary to carry out pre-breeding activities such as collecting and characterizing sorghum germplasm as a source of genetic diversity. It is anticipated that these activities will enable the identification of waxy types present in sorghum germplasm, which is needed by sorghum breeders. Presently, the Indonesian government has focused more on the management and conservation of the sorghum germplasm collection, including native and introduced sorghum accessions maintained in the Agricultural Gene Bank, Indonesian Agency for Agricultural Research and Development (IAARD), the Ministry of Agriculture. Pre-breeding initiatives could be supported by the availability of such sorghum germplasm to assist in the development of sorghum varieties with lower amylose content in Indonesia.

Identification of waxy phenotype in sorghum germplasm as donor parent in crosses is primarily needed to develop new sorghum variety possessing low amylose content. Identification of waxy sorghum can be conducted through molecular and qualitative approaches (Cho et al., 2015). Molecular based methods are carried out based on waxy allele-specific DNA marker, known as wx^a and wx^c alleles, whereas qualitative methods are performed based on iodine staining which can be carried out in pollen, endosperm, and sorghum grains (Pedersen et al., 2004; Cho et al., 2015; Firdaus et al., 2020). In this study, we were able to identify waxy sorghum at the genotype and phenotype levels by combining these two approaches, which is thought to be a more accurate strategy. Using waxy gene-based markers and iodine staining techniques, the current work sought to detect waxy characteristics in sorghum germplasm.

Results

Waxy alleles on germplasm and national varieties of sorghum

The results of molecular identification of waxy sorghum genotypes using two known specific primer sets for detecting waxy gene alleles (wx^a and wx^c) were presented in Table 1. Out of 27 introduced sorghum accessions amplified by genome-specific primer set for wx^a alleles none of them revealed to possess wx^a allele. In other words, they were all categorized as wild-type allele (*Wx*). On the other hand, 14 out of 27 introduced sorghum accessions amplified by specific primer set for wx^c allele possessed heterozygous waxy allele (wx^c/Wx), while the remaining 13 accessions showed wild-type alleles (*Wx*) (Table 1).

Among 21 local sorghum accession amplified by specific primer for wx^a allele, two accessions, including Demak 4 (Mejen) and Cantel Ketan were confirmed to carry the wx^a allele in heterozygous form (wx^a/Wx) (Fig 1), while the remaining 19 local sorghum accessions possessed the wild-type allele (*Wx*). Meanwhile, 8 out of 21 local sorghum accessions amplified by wx^c allele specific primer contained wx^c alleles in the form of heterozygous form (wx^c/Wx), while other accessions contained wild-type alleles (*Wx*) (Table 2). Fig 2 shows the banding pattern of wx^c allele (wx^c/Wx) with a thick and clear DNA band on Rumbia accession derived from Lampung Province. All national sorghum-released varieties used in the current study carried nonwaxy or wild-type alleles (*Wx*).

A waxy trait of sorghum germplasm and national varieties at the genotype level

Applying iodine staining method on pollen of all the sorghum genotypes showed two properties of sorghum including heterowaxy and nonwaxy. Heterowaxy sorghum was indicated by the presence of two colors on sorghum pollen after treatment with iodine staining, of which the blackish-brown and yellow-orange colors refer to nonwaxy sorghum (*Wx*) and waxy sorghum (*wx*) characteristic, respectively. Prior to the introduced sorghum accessions, we found 17 out of 27 accessions conferring heterowaxy properties on their pollens after treated by iodine staining (Supplementary Fig 1), while the remaining were nonwaxy characteristics (Table 1). Aside from this, iodine staining treatment on 21 local sorghum

Table 1. Waxy allele type and waxy pollen and endosperm trait of introduced accession and national sorghum varieties.

No.	Accessions name	Allele		Trait		No.	Accessions name	Allele		Trait	
		wx^a	*wx*^{c}	Pollen	Endosperm			*wx*^{a}	*wx*^{c}	Pollen	Endosperm
1.	8309/199026	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*	17.	ICSV 88013	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*
2.	Keris	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*	18.	ICSV 93036	*Wx*	*Wx*	*heterowaxy*	*nonwaxy*
3.	Keris M-3	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*	19.	ICSB 11	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*
4.	Badik	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*	20.	ICSB 70	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*
5.	Hegari Genjah	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*	21.	ICSV 93009	*Wx*	*Wx*	*heterowaxy*	*nonwaxy*
6.	867.032	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*	22.	K.905	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*
7.	867.161	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*	23.	IS 9302	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*
8.	M-2	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*	24.	IS 18551	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*
9.	M-4	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*	25.	IS 23509	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*
10.	TX623B	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*	26.	TU B7	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*
11.	431	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*	27.	5D X 160	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*
12.	CK.2	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*	28.	Numbu	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*
13.	CK.5	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*	29.	Kawali	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*
14.	UPCA-S1	*Wx*	*Wx*	*heterowaxy*	*nonwaxy*	30.	Bioguma 1	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*
15.	Gadam Human	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*	31.	Pahat	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*
16.	Wad Jabis	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*						

Table 2. Waxy allele type and waxy pollen and endosperm trait of local sorghum accession.

No.	Accessions name	Allele		Trait		No.	Accessions name	Allele		Trait	
		wx^a	*wx*^{c}	Pollen	Endosperm			*wx*^{a}	*wx*^{c}	Pollen	Endosperm
1.	Mutiara Kulonprogo L70	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*	12.	Coley	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*
2.	Kempul Putih 62 R6	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*	13.	Somalia / Sorgum Putih	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*
3.	Demak 1 (Gajah)	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*	14.	Kempul Putih 64 K6	*Wx*	*Wx*	*heterowaxy*	*nonwaxy*
4.	Demak 2 (Gajah)	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*	15.	Rumbia (Lokal Lampung)	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*waxy*
5.	Demak 4 (Mejen)	*wx*^{a}/*Wx*	*Wx*	*heterowaxy*	*waxy*	16.	Lokal Bima 3	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*waxy*
6.	Demak 5	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*	17.	Cantel Ketan	*wx*^{a}/*Wx*	*Wx*	*heterowaxy*	*waxy*
7.	Butter Ainarup 2	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*	18.	Sorgum Pulut	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*waxy*
8.	Butter Biara	*Wx*	*Wx*	*heterowaxy*	*nonwaxy*	19.	Batar Ainarup Mean 2	*Wx*	*Wx*	*heterowaxy*	*nonwaxy*
9.	Lepeng	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*	20.	Watar Solor MEA	*Wx*	*wx*^{c}/*Wx*	*heterowaxy*	*nonwaxy*
10.	Butter Krek	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*	21.	Sorgum Manis	*Wx*	*Wx*	*heterowaxy*	*nonwaxy*
11.	Selayer 1	*Wx*	*Wx*	*nonwaxy*	*nonwaxy*						

accessions resulted in 14 out 21 local sorghum accessions possessing heterowaxy features (Supplementary Fig 2) and the remaining 7 local sorghum accessions were categorized as nonwaxy type (Table 2). All the four national sorghum released varieties revealed nonwaxy properties after treated with pollens-iodine staining (Supplementary Fig 3).

Waxy trait in sorghum germplasm and national varieties at the phenotype level

In the present study, confirmation of waxy genotype detected at molecular level was also carried out using iodine staining of the endosperm and sorghum grains which classified as a phenotype waxy evaluation. All the introduced sorghum accessions treated by endosperm-iodine staining were classified as nonwaxy sorghum (Table 1), whereas 5 out of 21 local sorghum accessions, consisted of Demak 4 (Mejen), Rumbia (Lokal Lampung), Lokal Bima 3, Cantel Ketan, and Sorgum Pulut were categorized as waxy properties (Table 2). Similar to the introduced sorghum accessions, all the national sorghum released variety exhibited nonwaxy properties after endosperm-iodine staining treatment (Supplementary Fig 4). Based on the grains-iodine coloring treatment, we found that all introduced accessions were categorized as nonwaxy properties. In regards to the local sorghum accessions treated by grains-iodine staining, one local accession, named as Rumbia (Lokal Lampung) was confirmed to possess waxy type (Supplementary Fig 5).

Similar to the results with iodine staining on pollen and endosperm, all the national sorghum released varieties showed nonwaxy types after their grains were treated by iodine staining, demonstrating that the four sorghum varieties were confirmed to have nonwaxy type at both genotype and phenotype level.

Discussion

The two *wx* alleles, named as wx^a and wx^c alleles for the presence or absence of *GBSS I* protein in sorghum grain have been characterized and differed in their mutation forms. Mutations in the wx^a allele is occured due to the presence of large DNA insertions in the third exon of the *GBSS I* gene sequence (Sattler et al., 2009; Cho et al., 2015). Such mutations cause the loss of *GBSS I* activity which result in disruption of its gene expression at the transcription stage. On the other hand, the sequence mutation in the wx^c allele was caused by a point mutation in the area of splicing site at the intron 10 and exon 11 boundary of the *GBSS I* gene, a mutation that most likely resulted in the suppression of *GBSS I* gene expression. To be precise, this allele contains G to C replacement (Cho et al., 2015).

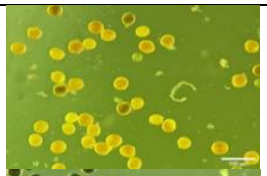
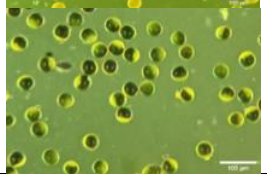
The diversity of waxy alleles observed in the sorghum germplasm was indicated by the detection of wx^a and wx^c alleles. Waxy alleles in the form of heterozygous type (*wx/Wx*), both in wx^a and wx^c alleles were categorized as heterowaxy sorghum (Sang et al., 2008). In contrast, those containing wild type alleles were categorized as the nonwaxy sorghum. Regarding to its phenotype performance, the sorghum heterowaxy will produce two types of grain, known as waxy and nonwaxy properties (Sang et al., 2008). The 48 sorghum accessions, including introduced and local accessions used in present study, contained 50% waxy alleles and 50% wild type alleles. Of these, introduced sorghum accession possessed only wx^c alleles, while local sorghum accessions contained both wx^a and wx^c alleles. These results would be useful for breeders to utilize the local sorghum accessions in sorghum breeding activities due to their greater waxy alleles diversity compared to those observed in the accessions of introduced sorghum.

Based on the results of waxy allele diversification across sorghum accessions, no accession containing both wx^a and wx^c alleles or in the other words waxy sorghum accession conferring wx^a allele was differed from accession containing wx^c allele. This finding suggest that the use of molecular marker to identify sorghum accession containing waxy alleles across sorghum core collection is proven to be an effective approach in sorghum breeding program. Previous study has also reported that application of molecular marker method is useful in sorghum breeding because plants can be selected at a very early stage (Sattler et al., 2009).

Iodine staining on sorghum pollens conducted in present study provided comprehensive information to classify waxy sorghum genotypes at the haploid level. In this regard, pollens with homozygous genotypes produce waxy (*wx*) or nonwaxy (*Wx*) pollen types, while those with heterozygous genotypes produces a mixture of waxy (*wx*) and nonwaxy (*Wx*) pollens. Classification of waxy genotypes at the haploid level is beneficial in the genetic study of sorghum germplasm because the breeding process will be more efficient (Pedersen et al., 2004).

The results of staining pollen on the detection of the waxy sorghum over 48 sorghum germplasm was in accordance with those of waxy allele identification method. In addition, the waxy genotypes detected at the pollen level were also aligned with the waxy allele, which is heterozygous. Rumbia (Lokal Lampung), Lokal Bima 3, and Cantel Ketan were found as sorghum accessions showing a larger composition of waxy pollen (*wx*) compared to nonwaxy pollen (*Wx*). These results suggest that the chance of waxy properties being expressed at a phenotype level is greater. This results also showed that seven sorghum accessions including UPCA-S1, ICSV 93036, ICSV 93009, Butter Biara, Kempul Putih 64 K6, Batar Ainarup Mean 2, and Sorgum Manis were classified as heterowaxy type. However, these sorghum accessions did not contain either wx^a or wx^c alleles or in the other words those possessed wild types alleles when they were subjected to PCR analysis.

Table 3. Alignment of waxy properties results in molecular methods and iodine staining.

No.	Trait	Accession name	Molecular	Pollen	Endosperm	Grain
1.	*Waxy*	Rumbia (Lokal Lampung)	*Heterowaxy (wx^c/Wx)*			
2.	*Nonwaxy*	Numbu	*Nonwaxy (Wx)*			

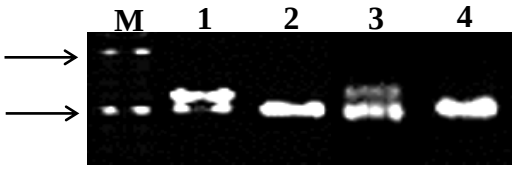


Fig 1. Identification of wx^a alleles on local sorghum accession. 1) Demak 4 (Mejen) accession contains heterozygous waxy allele (wx^a/*Wx*), 2) Demak 5 accession contains wild-type alleles (*Wx*), 3) Cantel Ketan accession contains heterozygous waxy allele (wx^a/*Wx*), and 4) Pulut Sorgum accession contains wild type alleles (*Wx*).

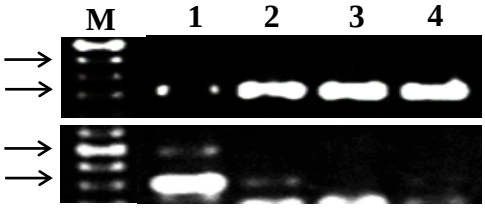


Fig 2. Identification of wx^c alleles on local sorghum accession. The upper panel of the *Wx* primer amplification and the bottom panel of the *wx* primer amplification. Accession Rumbia (Lokal Lampung) (No.1), Lokal Bima 3 (No.2), and Sorgum Pulut (No.4) contain heterozygous alleles (wx^c/*Wx*). Cantel ketan (No.3) contains wild-type alleles (*Wx*).

The seven accessions are thought to contain waxy alleles of other types besides wx^a and wx^c alleles.

Waxy properties are expressed in the coloring of endosperms and sorghum grains or at the level of fewer phenotypes than in the identification of waxy alleles and pollen coloring. This result is thought to be due to the detected sorghum plant genotype of a heterozygous type. Therefore, at the phenotype level not all grains produced are waxy but can also be nonwaxy (Sang et al., 2008). In addition, such results can be influenced by the level of expression of the *wx* gene and the composition of the *wx* gene in the grain endosperm. Accession of Rumbia (Local Lampung) at the molecular level has more DNA fragments that are amplified, so that at the phenotype level the waxy trait is detected.

Heterowaxy genotype in endosperm has two forms, namely *WxWxwx* and *Wxwxwx*. The composition of the *wx* gene determines the amylose content in sorghum grains (Sang et al., 2008). Heterowaxy sorghum with a lower number of *wx* genes than *Wx* genes was thought to have higher amylose content and, thus the iodine staining showed nonwaxy properties.

This phenomenon is in accordance with the principle of iodine staining on endosperm and sorghum grains in detecting waxy properties based on amylose content. Cho et al. (2015) reported that the red color of the sorghum grain showed amylose content in the range of 6.59-13.84%, while the dark blue color indicated an amylose content of 14.30-35.60%. The iodine staining method on pollen, endosperm, and grains showed consistent with the molecular method as presented in Table 3.

Identification of waxy allele genotypes gained information that the detected accession was not categorized as waxy but rather was as heterowaxy type. The amylose content (AC) in waxy sorghum is 0-2%, heterowaxy 3-20%, and nonwaxy >20% (Sang et al., 2008; Gerrano et al., 2014). Heterowaxy sorghum has an advantage over the health aspect of waxy sorghum due to its resistant starch composition (23.7%), while waxy sorghum is only 17.9% (Sang et al., 2008). Resistant starch can prevent diabetes by slowing the release of glucose in the blood and increase insulin sensitivity (Bojarczuk et al., 2022).

Table 4. Sorghum's waxy allele primer and PCR product size.

No.	Allele		*Primer Forward*	*Primer Reverse*	Size (bp)
1.	wx^a	wx^a	F: 5'- CGTGGCGAGATCAAACTCTA-3'	R: 5'-GCAGCTGGTTGTCCTTGTAG-3'	615
		Wx	F:5'-GGCCTGGATTCAATGTTCTT-3'	R: 5'-GCAGCTGGTTGTCCTTGTAG-3'	523
2.	wx^c	wx^c	F: 5'-GCTGGTTCTGAGTGCAACAA - 3'	R : 5'-ACTTCTTCTTGCCAGTGACG-3'	305
		Wx	F: 5'-GCTGGTTCTGAGTGCAACAA - 3'	R:5'-ACTTCTTCTTGCCAGTGACC-3'	305

Chen et al. (2019) reported that amylose content has a positive correlation with the potential yield. Therefore, heterowaxy sorghum possesses higher amylose content than waxy sorghum, having greater potential yields. In addition, the greater yield potential is influenced by the relatively stable production of heterowaxy sorghum amylose in climate conditions as diverse as stress due to high temperatures and water deficits (Yerka et al., 2016).

In the current study, a waxy property expressed at the phenotype level was only observed on the sorghum local accession. This data is important information for the development and utilization of Indonesia's local sorghum. The collection of sorghum germplasm recorded in the Sorghum Genetic Resources Passport Data Catalog amounts to 259 accessions consisting of introduced and local. In this study, only a total of 48 sorghum accessions (about 18.5%) of total accessions examined. It is believed that there is still the potential for waxy properties in accessions that have not been characterized yet. However, methods for detecting waxy sorghum in this study are recommended to be applied in characterizing other sorghum accessions by considering that these methods are specific. The results obtained are aligned, and it is easier and faster to detect sorghum accession with big population size.

Materials and Methods

Genetic materials

Genetic materials used in present study were 48 sorghum germplasm accessions consisted of 27 introduced and 21 Indonesian local sorghum accessions maintained at the Agricultural Gene Bank of Indonesian Agency for Agricultural Research and Development (IAARD) along with 4 national varieties of sorghum including Numbu, Kawali, Bioguma 1, and Pahat varieties.

DNA extraction

The sorghum leaves used for extracting the DNA of the sorghum genome were two-week-old (after planting). The CTAB method as described by Shehzad et al. (2009) was used in the DNA extraction of the sorghum genome.

PCR amplification of two waxy alleles

Two waxy alleles such as wx^a and wx^c analyzed in the plant genetic materials based on previous study conducted by Cho et al. (2015). The arrangement of primary nitrogen bases for detecting wx^a, wx^c, and wild-type *(Wx*) alleles and PCR product sizes was presented in Table 4. PCR analysis was performed in the final volume of 10 µL with a composition of 20 ng/µL DNA sample, 1× MyTaq Red Mix (Bioline Reagent Ltd., UK), 0.5 µM primer, and ddH_20. The PCR cycle consisted of predenaturation step at 95°C for 5 minutes, followed by 40 cycles of denaturation step at 95°C for 20 seconds, annealing step at 60°C for 30 seconds, and extension step at 72°C for 50 seconds. The final extension step was carried out at 72°C for 7 minutes. PCR products were separated on a 1.2% agarose gel (w/v) electrophoresis in a 0.5× TBE buffer and a 100 bp DNA ladder (Geneaid, Taiwan) was used to estimate PCR product sizes. DNA bands were visualized by UV illumination followed by photographed using a documentation gel tool (Gel Doc EZ Imager, BIO-RAD, California, US).

Iodine staining of sorghum pollen

Sorghum pollen staining was carried out based on the rapid iodine staining technique described by Pedersen et al. (2004) to identify waxy genotype in sorghum germplasm. Sorghum pollens were collected before anthesis period by shaking the panicles and accommodating the pollen using clean white paper. The collected pollens were inserted into a 1.5 mL microcentrifuge tube and successively suspended by adding 0.5 mL of 70% ethanol. The sample was then stored into the refrigerator at 4°C. In the next step, one drop of pollen suspension was dripped on a concave preparation glass followed by addition of a drop of Lugol solution (0.2% I_2–2% KI) (Merck, Germany). The stained suspension was placed on a microscope slide and observed under a light microscope (Olympus CX23, Japan) with a magnification of 10×. Pollen staining yellow-orange was scored as waxy type, whereas pollen staining blackish-brown was scored as nonwaxy. The above experiment was carried out three times replication to verify result.

Iodine staining of endosperm and sorghum grains

The method of endosperm staining was carried out according to endosperm staining method as described by Firdaus et al. (2020). Sorghum grains were split into two parts, of which one drop of Lugol solution (0.2% I_2–2% KI) (Merck, Germany) was dripped onto the

sorghum endosperm. The discoloration occurred in both endosperm was observed under a stereo microscope at a magnification of 20× (Olympus, Japan). Iodine staining of sorghum grains was carried out based on staining method described by Pedersen et al. (2004). One sorghum grain was placed in a microplate subjected to crush the grain coarsely. One milliliter of H_2O distilled water was added to each well of a microplate and the samples were heated in the oven at 95°C for 1 hour and followed starch gelatinization by cooling the samples at room temperature. Lugol solution (0.2% I_2–2% KI) was further dripped into each well and a color change was finally observed. Endosperm and sorghum grains in each well which categorized as waxy type were indicated by wells producing a reddish-brown color, while nonwaxy produced a purple or blackish-blue color. The above experiment was carried out three times replication to verify the result.

Conclusion

In the present study, waxy allele types observed in 48 sorghum accessions along with 4 national sorghum breeding varieties analyzed varied greatly. At the genotype level, a total of 17 introduced and 14 local sorghum accessions were detected as waxy type, whereas at the phenotype level, four local sorgum accessions named as Demak 4 (Mejen), Rumbia (Lokal Lampung), Lokal Bima 3, Cantel Ketan, and Sorgum Pulut were identified as waxy types. Those introduced and local sorghum genotypes categorized as waxy type, possessing heterozygous alleles in their genome reflecting heterowaxy sorghum type.

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