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# NOVEL NONWAXY ALLELE VARIATION AMONG FOXTAIL MILLET GENOTYPES FROM INDONESIA

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## SUMMARY

Foxtail millet (*Setaria italica* [L.] P. Beauv.) has a low glycemic index due to its high grain amylose content. The synthesis of amylose in endosperm tissue is controlled by a single dominant gene, *Waxy* (*Wx*), which encodes the granule-bound starch synthase I enzyme. The loss of function of this gene results in a waxy endosperm due to failure in amylose synthesis. Thirteen allele variations of *Wx* gene in foxtail millet that have been identified so far arise from transposable element (TE) insertions into the exonic or intronic region of the gene. The objective of this study was to identify allele variation in the *Wx* gene among Indonesian foxtail millet genotypes. The 21 foxtail millet genotypes examined in this study had nonwaxy endosperm. Three types of nonwaxy alleles were identified in these genotypes. Fourteen genotypes with nonwaxy endosperm were confirmed to be type I (wild type), and five other nonwaxy genotypes were confirmed to be type VI. One genotype with type VIII had waxy endosperm. All TE insertions shared the same sequences as previously reported TEs. Additionally, a novel polymorphism in the intron 12 region was identified. This polymorphism was generated by the partial deletion of TSI-10 and intron 12 from the type VI allele. The deletion was thought to be a result of double-strand break repair through the microhomology-mediated end joining pathway. Genotypes with this polymorphism retained their nonwaxy endosperm. This novel polymorphism was designated as a new *nonwaxy* allele type XI.

**Keywords:** Allele variation, foxtail millet, transposable element, waxy gene

**Key findings:** The *Wx* allele of foxtail millet genotypes from Indonesia was identified. A novel allele variation that corresponded to nonwaxy endosperm was observed among the genotypes. The novel allele is an important addition to *Wx* allele variations in foxtail millet.

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## INTRODUCTION

Approximately 66%–76% of cereal grains are composed of carbohydrates, with starch as the major type (Koehler and Wieser, 2013). Starch comprises two distinct polysaccharides: the linear chain amylose and the highly-branched amylopectin. Typically, amylose accounts for 15%–30% of grain starch (Hunt *et al*., 2010), although its content is highly variable among species and genotypes (Mohammadkhani *et al*., 1998; Udachan *et al*., 2012). The ratio of amylose and amylopectin directly affects the physicochemical properties of starch (Fredriksson *et al*., 1998), as well as the eating and cooking quality of grains (Pang *et al*., 2016). High-amylose grains generally become hard and fluffy after cooking, whereas low-amylose grains become soft and sticky (He *et al*., 2015). Waxy endosperm has a very low or nearly undetectable level of amylose (Nakayama *et al*., 1998). Amylose content has a negative relationship with glycemic index (GI) value (Fitzgerald *et al*., 2011). Foods with low GI value are especially beneficial for the prevention and management of type II diabetes (Thathola *et al*., 2010).

Foxtail millet (*Setaria italica* [L.] P. Beauv.), sometimes also known as Italian millet, has lower GI value than other non-millet cereals because of its high grain amylose content (He *et al*., 2015; Muthamilarasan *et al*., 2016). Amylose content in foxtail millet grains has been reported to be as high as 31.9%, although variations in amylose content exist among landraces (Nakayama *et al*., 1998). Millet grain protein ranges from 10.7% to 18.5% (Upadhyaya *et al*., 2011) and is higher than rice protein, which only reaches up to 6.8% (Muthamilarasan *et al*., 2016). Foxtail millet grains are also rich in minerals, such as calcium, iron, and zinc, which amount to 171.2–288.7, 58.2–68.0, and 54.5–74.2 mg $kg^{-1}$, respectively (Upadhyaya *et al*., 2011). This crop shows high tolerance to drought and salinity stress (Ardie *et al*., 2015; Lapuimakuni *et al*., 2018; Widyawan *et al*., 2019). Foxtail mill*et* also has a higher water use efficiency than maize; specifically, for each gram of dry biomass, foxtail millet only requires 257 g of water, whereas maize requires 470 g of water (Li and Brutnell, 2011). Foxtail mill*et* also can be cultivated in low-nutrient soil (He *et al*., 2015).

Granule-bound starch synthase I (GBSSI), encoded by the *Waxy* (*Wx*) gene, is an enzyme responsible for the synthesis and accumulation of starch granules in cereal endosperm. The gene encoding GBSSI is highly expressed in the endosperm, whereas GBSSII is found in leaf, culm, and pericarp tissue (Vrinten and Nakamura, 2000). The *Wx* gene has

been widely studied across cereal species, such as rice (Hirano *et al*., 1998), maize (Varagona *et al*., 1992), sorghum (Pedersen *et al*., 2005), wheat (Vrinten and Nakamura, 2000), and barley (Domon *et al*., 2002; Asare *et al*., 2012). In rice, a single nucleotide substitution at the 5′ splice site of *Wx*'s first intron causes the inefficient processing of pre-mRNA and results in a waxy phenotype (Hirano *et al*., 1998). Indels (Domon *et al*., 2002) and nonsynonymous single nucleotide polymorphisms (SNPs) (Asare *et al*., 2012) are found in *waxy* alleles in barley. Waxy endosperm in maize is caused by a disruption in gene expression due to transposable element (TEs) insertions within the gene (Varagona *et al*., 1992).

The complete sequence of *Wx* cDNA from foxtail millet has been successfully determined (AB089141) (Fukunaga *et al*., 2002). The *Wx* gene of foxtail millet consists of 14 exons, but only exons 2–14 contribute to the coding sequence (Fukunaga *et al*., 2002). This gene also shows high similarity with *Wx* genes from other cereal species (Shapter *et al*., 2009; Hunt *et al*., 2010). The allelic variations of the *Wx* gene in foxtail millet arise from independent insertions of TE into the coding or noncoding regions of genes (Fukunaga *et al*., 2002; Kawase *et al*., 2005). Fukunaga *et al*. (2002) identified seven restriction fragment length polymorphism (RFLP) classes. These seven classes correspond with the endosperm type of foxtail millet grains. Kawase *et al*. (2005) later developed those seven RFLP classes into 10 types and three subtypes alleles on the basis of a PCR amplification pattern using four specific primer pairs.

Allelic variations of *Wx* gene in various foxtail millet accessions from around the world have been reported by several research groups (Kawase *et al*., 2005; Hachiken *et al*., 2013; Kuo *et al*., 2018). Although this gene is well-studied, only a few foxtail millet genotypes from Indonesia were included in previous studies. The majority of the genotypes were from East Asia, followed by South and Southeast Asia, and a few from Central Asia and Europe (Fukunaga *et al*., 2002; Kawase *et al*., 2005; Van *et al*., 2008; Hachiken *et al*., 2013; Kuo *et al*., 2018). In this study, we aimed to determine the endosperm types and identify the *Wx* allele in 23 foxtail millet genotypes collected from Indonesia.

## MATERIALS AND METHODS

### Plant material

A total of 23 foxtail millet genotypes, including 13 landraces collected from various regions of Indonesia, were used in this study. Among the genotypes used in this study, two (ICERI 5 and ICERI 6) have been reported to show significant salinity and drought stress tolerance (Ardie *et al*., 2015; Lapuimakuni *et al*., 2018; Widyawan *et al*., 2019). Seeds from each genotype were subjected to the iodine test to assess the starch phenotype of the endosperm. Three seeds were cut in half and then immersed in Lugol's solution (UPOV, 2010). Endosperm color was observed under a stereomicroscope. A seed was also crushed between glass slides and stained with 100× freshly diluted Lugol's solution (Hunt *et al*., 2010). Three replicates of each genotype were observed under 10× objective

magnification with a microscope. Waxy and nonwaxy endosperms stained reddish-brown and dark blue, respectively.

**Molecular genotyping and sequence analyses**

Genomic DNA was extracted from the leaf tissue of 3-week-old foxtail millet seedlings via the CTAB procedure (Murray and Thompson, 1980) without the addition of 2-mercaptoethanol to the extraction buffer. Precipitated DNA was dissolved in 100–150 µL of TE buffer and stored at −20 °C until it was used. DNA concentration was estimated by using a Nucleic Acid Analyzer system (Medclub Scientific Co., Ltd, Taiwan). Four primer combinations, which were adopted from Kawase *et al*. (2005) (ex1/ex2, ex2int2/ex4r, M5/R7, and M7/R10), were used to identify the *Wx* allele of each genotype. PCR was performed with a 50 µL mixture containing 5× GoTaq Reaction buffer, 1 U GoTaq DNA polymerase (Promega, Madison, WI, USA), 50 pmol of each forward and reverse primers, 2 mM dNTPs mixture (Toyobo Co., Ltd, Japan), and 5 ng $\mu L^{-1}$ genomic DNA. For primer pairs with high annealing temperatures or amplicons larger than 1 kb, PCR amplification was performed by using KOD FX (Toyobo Co., Ltd, Japan) in accordance with the supplier's instructions, and the reactions were adjusted to 20 µL mixtures. PCR products were electrophoresed on 1.5% agarose gels, stained with ethidium bromide, and then visualized under a UV transilluminator. *Wx* allele was determined on the basis of the pattern of amplicon size from each primer pair (Kawase *et al*., 2005).

The PCR products of the M7/R10 primer set from representative allele types were purified by using Fast Gene Gel/PCR Extraction Kit (NIPPON Genetics, Japan) in accordance with the supplier's instructions. Purified products were sent to Macrogen (Japan) for direct sequencing. Direct sequencing reactions were also performed by Genome Lab DTCS Quick Start Kit (Beckman-Coulter Inc., USA) with a 10 µL reaction as described in the supplier's instructions. Sequence data from forward and reverse primers were edited and assembled with Geneious 11.0.5. Ambiguous bases were adjusted manually by comparing chromatogram data. Sequence alignment was performed by using MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/), and the result was adjusted manually.

**RESULTS**

**Identification of endosperm type and *Wx* allele in 23 Indonesian foxtail millet genotypes**

A simple iodine test was used to determine endosperm type by immersing halved grains and crushed seeds in Lugol's solution. Depending on the presence or absence of amylose in the starch endosperm, Lugol's solution could react to starch, resulting in a distinguishable color. Amylose reacted with iodine in Lugol's solution to provide a dark blue color, which indicated that the grain had a nonwaxy endosperm. Meanwhile, the absence of the dark blue color indicated a waxy type endosperm. The

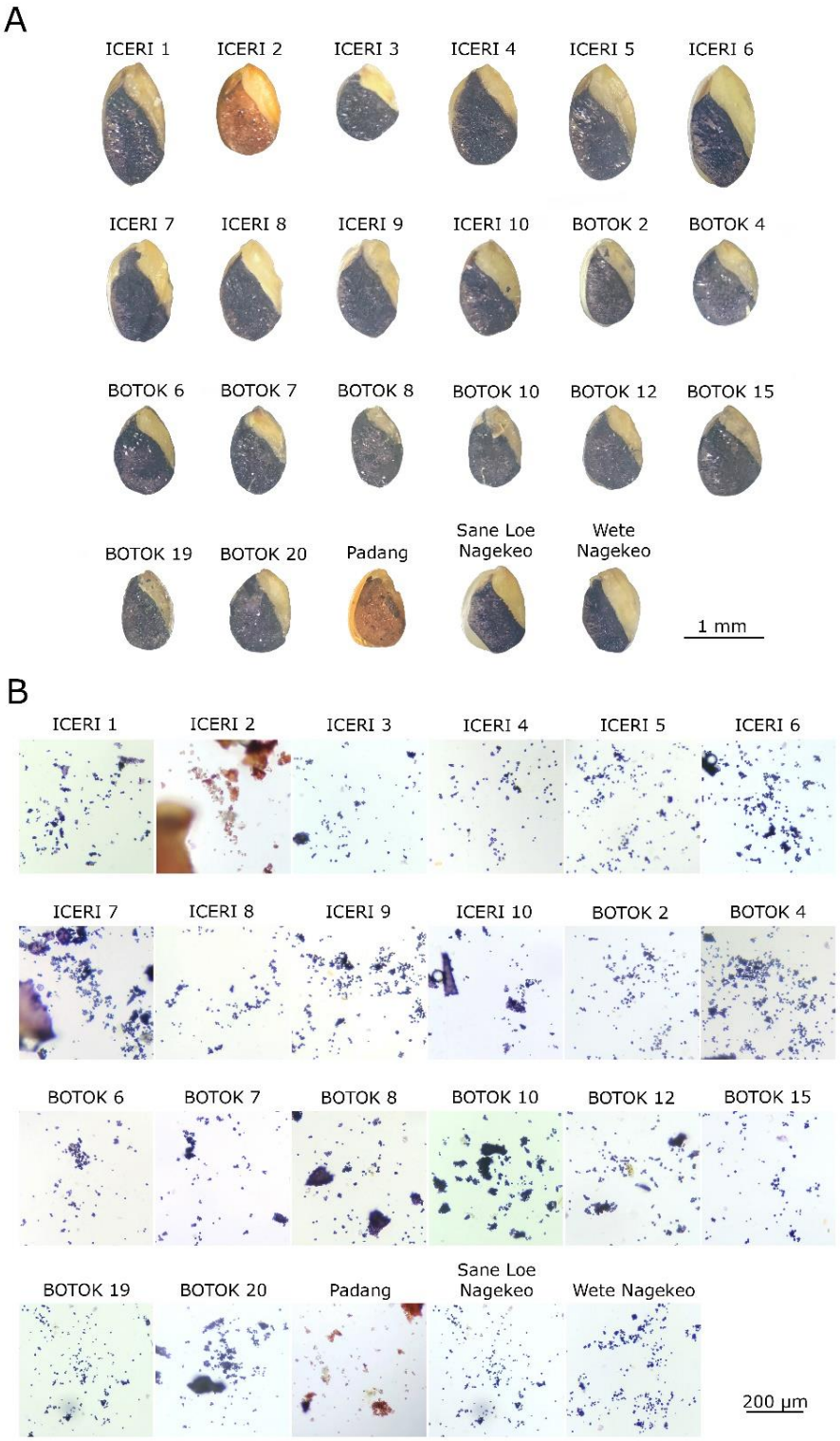


**Figure 1.** Foxtail millet seeds stained with Lugol's solution. (A) Method based on UPOV (2010) and (B) Hunt *et al*. (2010). Both methods produce similar results. Nonwaxy and waxy endosperm stained blue and reddish-brown, respectively.

21 genotypes tested in this study were stained dark blue by Lugol's solution, indicating the presence of amylose in the endosperm (nonwaxy) (Figure 1). Only two genotypes, ICERI 2 and Padang, had waxy endosperms, as shown by their reddish-brown color. Both staining methods enabled the consistent identification of every tested genotype. The half-seed of nonwaxy endosperms showed a uniform dark blue color (Figure 1A), whereas the blue color appeared purplish under a 10× objective magnification microscope (Figure 1B).

Four specific primer combinations were applied to identify the *Wx* allele type of each genotype. All four primer combinations, i.e., ex1/ex2, ex2int2/ex4r, M5/R7, and M7/R10, amplified different regions of the gene where TE insertions could be found. The wild-type amplicons of each primer set were expected to be ±850, ±400, ±800, and ±500 bp. Length polymorphisms observed from each primer set were found as a result of the mutational events experienced by the wild-type allele (type I) because of TE insertions. The *Wx* allele type from each genotype could be determined by analyzing the pattern of amplicon sizes from each primer set.

In this study, only primer pair M7/R10 showed polymorphisms among genotypes, whereas the remaining primer pairs produced wild-type bands (Table 1; Figure 2). Fourteen genotypes produced ±500 bp-sized amplicons, whereas primer sets ex1/ex2, ex2int2/ex4r, and M5/R7 produced wild-type bands. This pattern represented allele type I. A ±1000 bp-sized amplicon produced from M7/R10 amplification by five genotypes and wild-type amplicons from the remaining primer sets represented type VI. Only one genotype produced a ±2000 bp-sized amplicon from primer pair M7/R10 and a wild-type amplicon for the other three primer pairs. This particular pattern represented allele type VIII. Among the three allele types identified thus far, only type VIII was associated with waxy endosperm (Table 1; Figure 1). The results for allele identification were also in agreement with the iodine test results.

Interestingly, in this study, two genotypes with nonwaxy endosperm (Botok 2 and Botok 4) produced a ±700 bp-sized band through M7/R10 amplification (Figure 2). The primer pairs ex1/ex2, ex2int2/ex4r, and M5/R7 produced wild-type bands. The size of the amplicon produced with M7/R10 deviated from that of the thirteen alleles described previously by Kawase *et al*. (2005). The amplification pattern from four primer pairs did not match any of the *Wx* allele types. This is a unique finding given that it has been unreported thus far. We were unable to determine the allele type of Padang, one of the two known waxy genotypes, due to unsuccessful PCR amplification attempts with the primer set of M5 and R7.

**Sequence analyses of TE insertions in the M7/R10 region**

Although the *Wx* allele variations observed in type VI and type VIII were caused by TE insertions in intron 12 (Kawase *et al.* 2005), the length polymorphism observed in Botok 2 and Botok 4 was unknown. The sequences of the M7/R10 region were evaluated in two genotypes from each allele type, except for type VIII. ICERI 5 and ICERI 6 were chosen to represent type I, ICERI 7 and ICERI 9

**Table 1.** Genotypes and their origins, endosperm types, PCR amplification of four primer sets, and corresponding allele types

| Geno-types | Origin[1] | Endo-sperm type | Amplicon size | | | | Allele type |
|---|---|---|---|---|---|---|---|
| | | | ex1/ex2 | ex2int2/ ex4r | M5/R7 | M7/R10 | |
| ICERI 1 | ICERI | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±500 bp | I |
| ICERI 2 | ICERI | Waxy | ±850 bp | ±400 bp | ±800 bp | ±2000 bp | VIII |
| ICERI 3 | ICERI | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±500 bp | I |
| ICERI 4 | ICERI | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±1000 bp | VI |
| ICERI 5 | ICERI | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±500 bp | I |
| ICERI 6 | ICERI | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±500 bp | I |
| ICERI 7 | ICERI | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±1000 bp | VI |
| ICERI 8 | ICERI | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±1000 bp | VI |
| ICERI 9 | ICERI | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±1000 bp | VI |
| ICERI 10 | ICERI | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±1000 bp | VI |
| Botok 2 | Labapu, NTT | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±700 bp | XI |
| Botok 4 | Labapu, NTT | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±700 bp | XI |
| Botok 6 | Labapu, NTT | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±500 bp | I |
| Botok 7 | Labapu, NTT | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±500 bp | I |
| Botok 8 | Labapu, NTT | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±500 bp | I |
| Botok 10 | Labapu, NTT | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±500 bp | I |
| Botok 12 | Alata, NTT | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±500 bp | I |
| Botok 15 | Alata, NTT | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±500 bp | I |
| Botok 19 | Alata, NTT | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±500 bp | I |
| Botok 20 | Alata, NTT | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±500 bp | I |
| Padang | Sumatera | Waxy | ±850 bp | ±400 bp | n/a[2] | ±500 bp | - |
| Sane Loe Nagekeo | Nagekeo, NTT | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±500 bp | I |
| Wete Nagekeo | Nagekeo, NTT | Nonwaxy | ±850 bp | ±400 bp | ±800 bp | ±500 bp | I |

1 ICERI, Indonesian Cereals Research Institute; NTT, Nusa Tenggara Timur
2 n/a, not amplified

were used to represent type VI, ICERI 2 was selected to represent type VIII, and Botok 2 and Botok 4 were chosen to represent the novel polymorphism. M7/R10 sequences from ICERI 5 and ICERI 6, both type I, were found to be identical to the Yugu-1 (type I) sequence taken from the Genbank database (Figure 3). A short interspersed nuclear element (SINE) family retrotransposon inserted into intron 12 and was flanked by a 14 nucleotide-long target site duplication (TSD) (Figure 3; Figure 5A) in ICERI 7 and ICERI 9. Multiple sequence alignment revealed a seven poly-T tail, a known characteristic of SINE retrotransposons, w at the nucleotide position of 363 (Figure 3).

This mutational event resulted in the type VI allele. The TE sequences in ICERI 7 and ICERI 9 were completely identical to each other. In addition, both sequences shared the same sequence as the previously reported TSI-10 (AB210219). As for type VIII, we were unable to obtain the full sequence of the M7/R10 region from ICERI 2. However, we could confirm that the partial TE sequence

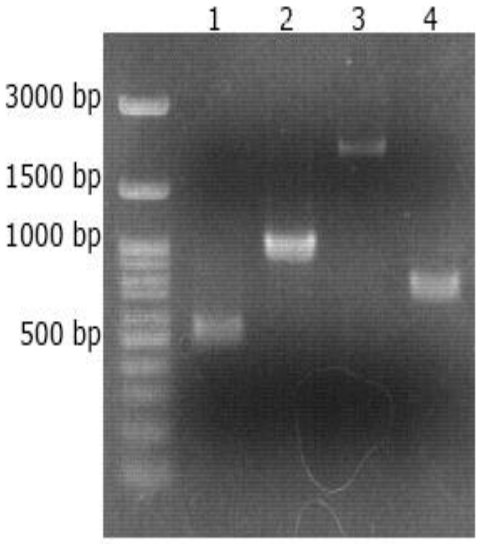


**Figure 2.** PCR polymorphisms observed from M7/R10 primer pair amplification. Lanes 1, 2, 3, and 4 represent type I, VI, VIII, and XI, respectively.

from ICERI 2 shared the same sequence as TSI-11 (AB210220) (Figure 4).

Both M7/R10 regions of Botok 2 and Botok 4 shared identical sequences (Figure 3). Multiple sequence alignment revealed that the novel polymorphism observed in Botok 2 and Botok 4 was caused by the partial deletion of the type VI sequence (Figure 3; Figure 5). This deletion spanned 241 nucleotides, i.e., 184 nucleotides from TSI-10 and 57 nucleotides from intron 12. We identified the presence of two identical short sequences, 5'–CTCCTT–3', which flanked the deleted region (Figure 3; Figure 5B). Deletion in the type VI allele was most likely a result of double-strand break (DSB) repair through the microhomology-mediated end joining (MMEJ) pathway (Figure 5). After the occurrence of DSB, DNA-end resection generates a long 3' single-stranded DNA tail. Microhomology search is initiated at the MMEJ repair mechanism, and DNA breaks are ligated at microhomology sites (Figure 5B). From these results, we concluded that the amplification pattern observed in Botok 2 and Botok 4 was associated with a novel *nonwaxy* allele of foxtail millet. We then designated this allele as a type XI allele.

## DISCUSSION

A simple iodine test using Lugol's solution is a common method for detecting the presence of starches in organic compounds; depending on the starch composition, Lugol's solution can react to starch, resulting in a distinguishable color (Serna-Saldivar 2012). The reaction between iodine and amylose produces a dark blue color. By contrast, amylopectin has a weak affinity for iodine, thus producing reddish color upon contact (Serna-Saldivar 2012). Amylose-

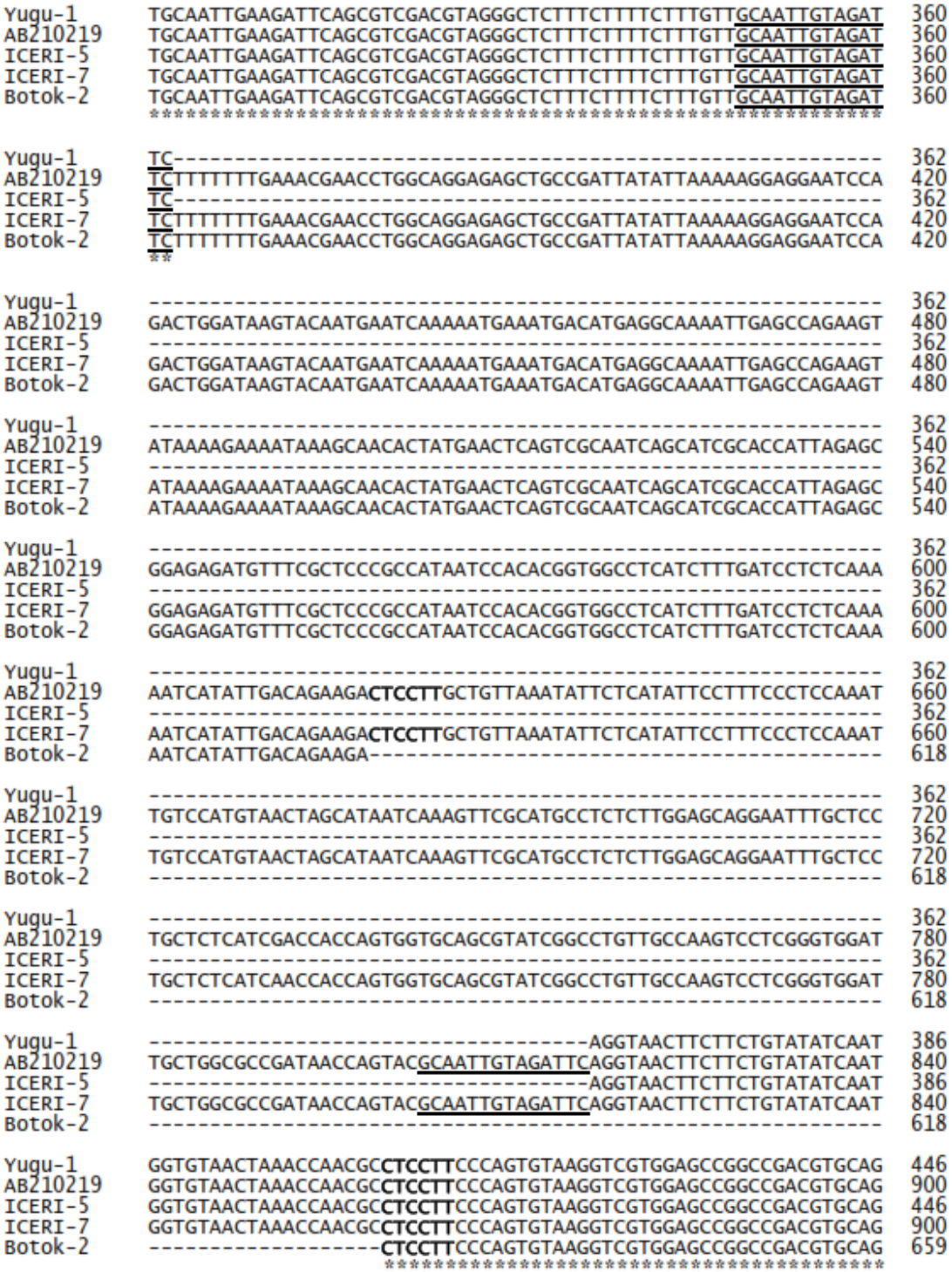


**Figure 3.** DNA sequences of insertion regions from ICERI 5 (type I), ICERI 7 (type VI), and Botok 2 (type XI) compared with those from Yugu-1 (type I) and TSI-10 (AB210219). Asterisks (*) indicate identical bases; minus (−) signs indicate missing nucleotides; underlined letters indicate target site duplication sequences; bold letters indicate putative microhomology sequences.


**Figure 4.** Comparison of the partial sequence of ICERI 2 (type VIII) insertion and TSI-11 (AB210220) showing that both insertions share identical sequences. Asterisk (*) indicates identical bases; minus (−) sign indicates missing nucleotides.

containing starch is further classified as nonwaxy, and starch with an undetectable level of amylose is classified as waxy.

The amylose content of seed endosperm can be altered due to various mutation events, such as insertions and/or deletions, experienced by the *Wx* gene (Domon *et al*., 2002; Sattler *et al*., 2009; Hunt *et al*., 2010), SNPs (Hirano *et al*., 1998; Asare *et al*., 2012), and TE activity (Umeda *et al*., 1991; Varagona *et al*., 1992; Fukunaga *et al*., 2002). In the case of foxtail millet, TE activity is responsible for the changes in *Wx* gene expression (Fukunaga *et al*., 2002; Kawase *et al*., 2005). Thirteen allele variations arose from various TE insertions into exon or intron regions that were followed by deletions, nested insertions, and/or intragenic recombination (Kawase *et al*., 2005). Various TEs can be found within intron 1, exon 3, exon 10, and/or intron 12.

The waxy endosperm of foxtail millet might have evolved from a nonwaxy genotype after domestication. The wild progenitor of foxtail millet, *Setaria viridis* Beauv., has a nonwaxy endosperm only (Nakayama *et al*., 1998). Furthermore, *Wx* sequences from nonwaxy genotypes are highly diverse (Kawase *et al*., 2005; Hachiken *et al*.,

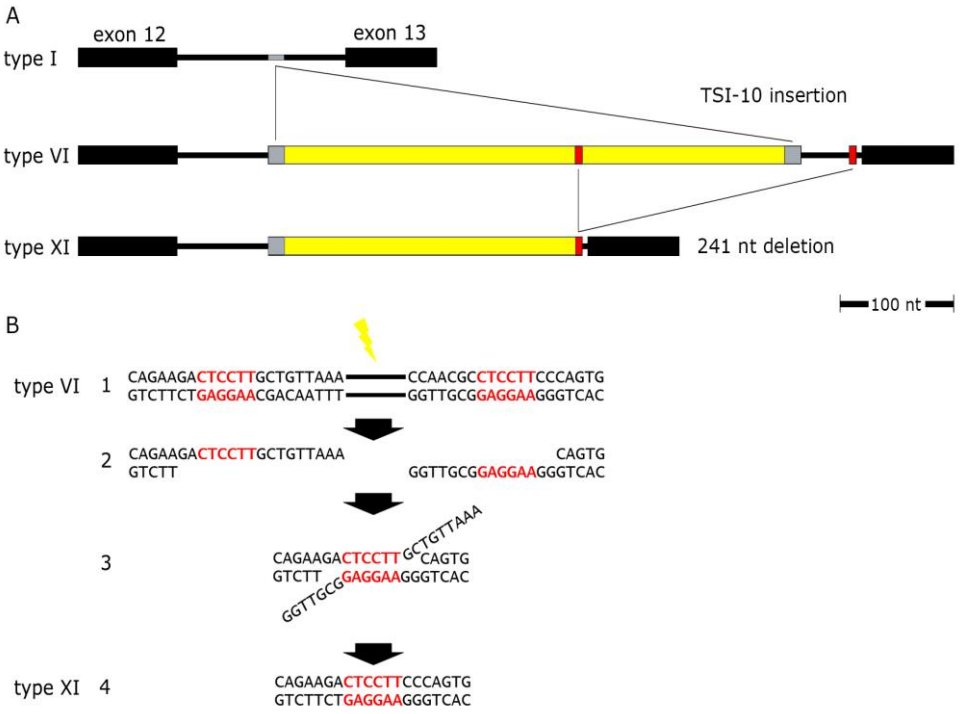


**Figure 5.** Emergence of type VI and XI alleles from type I via insertion and deletion events. (A) TSI-10 (yellow box) was inserted between TSD (grey box), generating a type VI allele. The deletion of 241 nucleotides flanked by short microhomologous sequence (red box) from type VI gives rise to type XI. (B) Deletion due to DSB repair through the MMEJ pathway, adopted from van de Lagemaat *et al*. (2005). Error-prone repair mechanism involves (1) a double-stranded DNA break in type VI, (2) DNA-end resection generating a long 3′ single-stranded DNA tail, (3) microhomology search, and (4) ligation. Repair mechanism generates 241-bp deletion mediated by a 6-bp microhomology.

2013). In the 10 types and three subtypes described by Kawase *et al*. (2005), seven allele variations correspond to waxy endosperm. These seven allele variations arose from large TE insertions, nested insertions, or TE insertions into exonic regions.

The *Wx* allele type can be determined on the basis of PCR polymorphisms by using four specific primer pairs (Kawase *et al*., 2005). Depending on the amplicon size of a particular primer pair, allele type can be easily assigned to one of the 13 alleles described by Kawase *et al*. (2005). Three out of 13 allele types were found among the genotypes used in this study (Table 1). All PCR amplicons with the expected sizes were produced by four specific primer sets in nonwaxy genotypes, which were designated as type I (wild type). The wild-type allele was most abundant among nonwaxy genotypes in this study (Table 1). This allele is also most prevalent worldwide (Kawase *et al*., 2005; Hachiken *et al*., 2013). Other *nonwaxy* alleles found in

this study were type VI and the novel type XI. The only *waxy* allele among 22 genotypes was type VIII, which had the largest TE insertion (Figure 2).

All four allele types observed in this study arose from variations within intron 12 (Table 1; Figure 2). Large variations in this region are common in *Wx* genes across grass species due to insertion and/or deletion events (Shapter *et al*., 2009). The insertion of TSI-10, a SINE family member (Kawase *et al*., 2005; Hirano *et al*., 2011), into intron 12 of type I gave rise to type VI. Genotypes with type VI alleles retain their nonwaxy endosperm. All type VI alleles, including those in this study, share the same insertion (Kawase *et al*., 2005; Van *et al*., 2008). Type VI is predominant among genotypes from South and Southeast Asia (Kawase *et al*., 2005; Van *et al*., 2008; Hachiken *et al*., 2013). Other nonwaxy allele types, such as type II, are observed in genotypes from Nepal and Kenya (Kawase *et al*., 2005). This study, so far, is the only study that mentioned this allele type. Genotypes with type III alleles originate from Japan (Kawase *et al*., 2005), Taiwan, and the Philippines (Kawase *et al*., 2005; Hachiken *et al*., 2013; Kuo *et al*., 2018). Type IX is present in genotypes from Taiwan (Hachiken *et al*., 2013; Kuo *et al*., 2018). Kawase *et al*. (2005) also recorded the presence of type IX in genotypes from the Philippines and India.

Many SINE families are known to have a preference for gene-rich, GC-rich genomic regions (Weiner, 2002). TE insertions can cause alterations in gene expression (Weiner, 2002). Some SINE families show an association with certain plant genes (Tsuchimoto *et al*., 2008; Seibt *et al*., 2016; Keidar *et al*., 2018). SINEs are frequently found in intron and untranslated regions of genes (Seibt *et al*., 2016) but are rarely found in the exon (Tsuchimoto *et al*. 2008). The presence of SINE retrotransposons can play an important role in the structural variation of genes and genomes (Seibt *et al*., 2016; Keidar *et al*., 2018).

The results of the iodine test revealed that among the 23 genotypes, only two were waxy genotypes. However, type VIII in ICERI 2 was the only *waxy* allele that we were able to identify. We were unable to determine the allele type of Padang, which also had waxy endosperm. The additional insertion of TSI-11 into TSI-10 in type VI gave rise to type VIII, producing an approximately 1500 bp-long insertion (Figure 4). Both TEs were a class of SINE retrotransposons (Kawase *et al*., 2005; Hirano *et al*., 2011). This nested insertion further disrupted *Wx* gene expression and resulted in waxy endosperm (Kawase *et al*., 2005). Thus far, the type VIII allele is found only among Indonesian waxy genotypes (Kawase *et al*., 2005; Van *et al*., 2008; Hachiken *et al*., 2013). Other *waxy* alleles, such as type IV and type V, are more common among East Asian genotypes (Fukunaga *et al*., 2002; Kawase *et al*., 2005; Hachiken *et al*., 2013; Kuo *et al*., 2018). Subtypes IVa and IVb are found only among genotypes from Japan (Kawase *et al*., 2005). Waxy type VII is found among genotypes from East and Southeast Asia (Kawase *et al*., 2005; Hachiken *et al*., 2013). Type X allele with a waxy phenotype is found only in genotypes from Japan (Kawase *et al*., 2005).

Type XI from Botok 2 and Botok 4 emerged through the deletion of partial TE and intron 12 of type VI. Deletion can occur when DSBs, a common DNA damage, are imperfectly repaired (van de Lagemaat *et al*., 2005; McVey and Lee, 2008). Although the exact manner of the deletion in Botok 2 and Botok 4 was unknown, we strongly suspect that the deletion was a result of the MMEJ repair pathway. The MMEJ repair pathway relies on the alignment of short microhomologous sequences (2–25 nucleotides) at breakpoint junctions (McVey and Lee, 2008). This repair pathway always results in sequence deletion, which can span from a few hundred to thousands of bases, depending on the distance between two microhomology sites (McVey and Lee, 2008). MMEJ is also associated with chromosome rearrangements (Yu and Gabriel, 2003), and TE removals from genomes with TSDs serving as microhomology sites (van de Lagemaat *et al*., 2005). Although deletion did not alter the endosperm type given that both genotypes retained their nonwaxy endosperm, this novel polymorphism could become an important addition to existing *Wx* allele variations in foxtail millet.

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