1 **Variability in amylose content of Bangladeshi rice cultivars due to uniqueSNPs in *Waxy* allele**

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17 **List of Abbreviations**

- 18 AC = Amylose content
- 19 bp =Base pairs
- 20 cDNA = Complementary DNA
- 21 CTAB =Cetyltrimethylammonium bromide
- 22 DNA =Deoxyribonucleic acid
- 23 dNTP =Deoxynucleoside triphosphate
- 24 ESE = Exonic splicing enhancer
- 25 GBSS-I = Granule-bound starch synthase I
- 26 G3PDH = Glyceraldehyde-3-phosphate dehydrogenase
- 27 indel =Insertion/deletion
- 28 kb = Kilobase pairs
- 29 mM =Millimolar
- 30 mRNA = Messenger RNA
- 31 ng = Nanogram
- 32 pmol = Picomole
- 33 QTL = Quantitative trait loci
- 34 RNA = Ribonucleic acid
- 35 RT-PCR = Reverse transcription polymerase chain reaction
- 36 SF2/ASF = Splicing factor-2/ alternative splicing factor
- 37 SNP = Single nucleotide polymorphism
- 38 UTR =Untranslated region
- 39 $W_x = W_{axy}$
- 40 μl = Microlitre
- 41 μM = Micromolar
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46 **Abstract**

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48 *Waxy* gene (Granule Bound Starch Synthase I) is responsible for amylose synthesis in the rice
49 endosperm. Several mutations in this gene have been shown to be responsible for variable amylose
50 content (AC) phenotypes. The G/T mutation in 5' splice site of *Waxy* intron 1 has been traced as the
51 origin of the glutinous rice phenotype and differentiates low AC from non-glutinous intermediate/high
52 AC rice. Sequencing of *Waxy* promoter and 5' noncoding regions from 22 rice cultivars showed that
53 the evolutionary pattern of all Bangladeshi non-glutinous and most glutinous rice accessions are in
54 line with the general patterns of South and Southeast Asia. However, three cultivars Khara Beruin,
55 Modhu Beruin White and Kathali Beruin Red with low to very low amylose lacked the G/T splice site
56 mutation. These were more closely related to non-glutinous cultivars based on their SNP patterns in
57 promoter and noncoding regions. Further sequencing revealed a unique C deletion at a pyrimidine
58 tract of intron 5 of these three cultivars that may cause slippage of intron splicing. Additional SNPs at
59 intron 9 and 10 were also identified among these cultivars. These Bangladeshi-genotype-specific
60 mutations could be the cause of waxy or low amylose phenotypes in these glutinous accessions.

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62 **Key words:** Amylose content; *Waxy* gene; Glutinous and non-glutinous rice; SNPs

63 1. Introduction

64 Starch in rice endosperm contains amylose and amylopectin polysaccharides. Amylose is principally a
65 linear molecule containing $\alpha(1\rightarrow4)$ linked glucose units and makes up approximately 0-30% of total
66 starch. In contrast amylopectin is a branched molecule which contributes to approximately 70-100% of
67 total starch in rice endosperm (Martin and Smith, 1995). Higher amylose levels (20–30%) are
68 observed in many *indica* rice varieties in South Asia (Morishima et al., 1992). Lower amylose levels
69 (10–20%) are more common in *japonica* varieties that predominate in East Asia (Yamanaka et al.,
70 2004). Amylose content (AC) can be thus used to classify milled rice samples into different categories
71 such as glutinous/waxy (0–5% amylose), low AC (6–18%), intermediate AC (19–23%), or high AC
72 (>23%) types (Bergman et al., 2004). Low AC is usually associated with tender, cohesive, and glossy
73 cooked rice while high AC is associated with firm, fluffy, and separated grains in cooked rice (Juliano
74 et al., 1981). Therefore, AC is considered as one of the major characteristics for assessing rice cooking
75 and eating qualities. In addition, low AC rice varieties have long been used as a tool of improving
76 grain quality through conventional breeding as they represent an intermediate type between glutinous
77 and non-glutinous rice varieties (Dong, 2000; Sato et al., 2002).

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79 Genetic and molecular marker-based QTL analyses have revealed that the wide range of AC
80 variation in rice endosperm is mainly controlled by a major locus (*Wx* or *Waxy* gene) and multiple
81 minor loci (Fan et al., 2005; Inukai et al., 2000). The *Wx* gene in rice encodes the granule-bound starch
82 synthase I (GBSS-I) enzyme responsible for amylose biosynthesis (Okagaki and Wessler, 1988). The
83 origin of the glutinous phenotype such as those found in *japonica* rice has been traced to the *Wx^b*
84 allele, which contains a G/T mutation in intron 1 splice donor site of the *Wx* gene (Olsen and
85 Purugganan, 2002). Presence of this splice donor site mutation leads to inefficient splicing of *Wx* pre-
86 mRNA followed by reduced expression of functional GBSS-I (Cai et al., 1998). In contrast, non-
87 glutinous phenotype observed in many *indica* rice varieties has been traditionally associated with
88 *Wx^a* allele, which lacks the intron 1 G/T mutation. However many Northeast Asian rice varieties
89 carrying this intron 1 G/T mutation still show non-glutinous phenotype. This suggests that partial
90 suppression of the intron 1 G/T mutation may have an important role in the development of non-

glutinous rice of this region(Olsen and Purugganan, 2002). Amylose content of rice endosperm is therefore a major domestication trait that has evolutionary importance. Among the minor alleles affecting amylose content, a polymorphic CT_n microsatellite in the 5'-untranslated region (UTR) of *Wx* exon 1 has been shown to be correlated with various AC classes(Ayres et al., 1997). Other SNPs in coding regions of different *Wx* alleles lead to decreased AC by dropping the binding efficiency of GBSS-I to starch granules, changing amino acid at respective sites(Liu et al., 2009; Sato et al., 2002) and altering expression pattern of GBSS-I in endosperm and anthers(Mikami et al., 1999). In addition to *Wx*, mutations in genes such as *Dull*(Zeng et al., 2007) and *shrunk*(Asaoka et al., 1993) also indirectly affect AC in rice.

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Bangladesh is the world's fourth largest rice producing country. It is considered as an enriched rice germplasm reservoir with over 6500 wild, landraces and modern high yielding varieties (Elias et al., 2011). Majority of the rice species in Bangladesh are *indica* varieties with high amylose content and non-glutinous phenotype (Olsen and Purugganan, 2002). Several glutinous rice varieties, which are locally known as 'Beruin' or sticky rice, are also cultivated in the Northeastern region of Bangladesh(Dipti et al., 2003). These Beruin cultivars generally have low amylose content and are popular for making traditional dishes on special occasions. However the molecular basis of low amylose content in these local Beruin varieties is unknown. In this study we examined the nucleotide diversity at the *Wx* locus of selected Bangladeshi glutinous Beruin and non-glutinous rice cultivars to identify the underlying cause of AC variation in these genotypes. We also compared these Bangladeshi varieties with known *Wx* haplotypes in an attempt to understand their evolutionary pattern across Asia.

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114 **2. Materials and Methods**

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116 **2.1. Plant materials**

Seeds of eleven Beruin cultivars, which are representative of the diverse traditional landraces unique to the Northeastern region of Bangladesh (Sylhet), were collected from the local farmers. An equal

number of non-glutinous cultivars was then selected to allow identification of SNPs underlying the glutinous phenotype in Bangladeshi cultivars (Fig. 1A and 1B). Nine of the selected non-glutinous cultivars were from the same region as the Beruin cultivars. Two other popular aromatic cultivars, Kalijira and Kataribhog, were collected from the Northwestern region of Bangladesh (Dinajpur) to expand the genetic diversity of sampled non-glutinous cultivars. All of the selected cultivars are traditional transplanted Amanor monsoon season varieties. The collected seeds were multiplied during the planting season in the net house. Three plants of each cultivar were grown in a single pot to collect immature seeds.

2.2. Estimation of amylose content

The amylose content (AC) of all cultivars was determined according to previously published protocol (Juliano et al., 1981). Nazirshail rice flour (AC 25%, Bangladesh Rice Research Institute) was used as a control and potato starch flour (E. Merck, Germany) was used for generating the standard curve.

2.3. Genomic DNA Extraction

Genomic DNA was extracted from leaves of 3-week-old pooled plants (10-12 plants per cultivar) using CTAB extraction method and in some cases by using the Qiagen DNeasy® plant mini kit. DNA of all cultivars was quantified by spectrophotometry. Quality of extracted DNA was checked by 1% agarose gel electrophoresis and compared with known concentration of Lambda DNA (Invitrogen, USA).

2.4. PCR amplification and DNA sequencing

A 2.7 kb upstream regulatory region of the *Wx* gene including promoter, exon 1, intron 1 and part of exon 2 was PCR amplified and sequenced in all 22 Bangladeshi rice cultivars. PCR amplification was performed in two steps to amplify overlapping ~1.5 kb segments of the above upstream regulatory region using primers described by Olsen and Purugganan (2002) (Supplementary Table 1). For each primer set, 15 µl PCR reaction mixture containing 60 ng of DNA template, 1 × PCR buffer, 1.67 mM MgCl₂, 100 µM of each dNTP, 0.33 pmol Forward /Reverse primer, 1 unit Taq DNA polymerase and

0.1 unit Pfx® DNA polymerase (Invitrogen, USA) was used. Amplification conditions were 94 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 65.2 °C for 1 min, 72 °C for 1 min and a final extension of 72 °C for 8.30 min.

For further characterization of the *Wx* coding region in Shamudrophena, Kathali Beruin Red, Modhu Beruin White, Khara Beruin and Mou Beruin, two different segments were amplified and sequenced using four sets of primers (Supplementary Table 1). The first segment (~0.2 kb) included part of exon 2, which is known to contain a 23 bp duplication in some glutinous varieties. This segment was amplified using primer pair sequences Glu-23F and Glu-23R as described by Wanchana et al. (2003). The other ~2kb segment including exons 3-10 was amplified using three overlapping primer pairs (Supplementary Table 1). These primers were designed using primer3 program (<http://frodo.wi.mit.edu/primer3/>) based on sequence of Nipponbare *Wx* locus (OS06G0133000) in Gramene database (www.gramene.org). PCR amplification conditions were the same as for the *Wx* upstream regulatory region except the annealing temperature, which was optimized for each primer pair (Supplementary Table 1).

All PCR amplicons were purified using QiAquick gel extraction kit (Qiagen, Germany) and directly sequenced in ABI 3730XL genetic analyzer using both forward-reverse and internal primers (Supplementary Table 1). Each fragment was sequenced twice for SNP confirmation. All of the sequences from this study have been deposited in GenBank (accession no. JF834042.1-JF834063.1 and KP675770-KP675774).

2.5. Sequence analysis

Wx upstream regulatory region sequences from 22 Bangladeshi rice landraces were compared to 18 previously reported haplotypes across Asia (Genbank accessions AY136760–AY136784). All multiple sequence alignments were generated using ClustalW (Thompson et al., 2002). SNP/indels were identified using TASSEL (Bradbury et al., 2007). Neighbour-joining tree of aligned *Wx* upstream regulatory region (2.7 kb) sequences was constructed using MEGA 6.06 software package (Tamura et

al., 2013) with Kimura two-parameter model and complete deletion of alignment gaps. Bootstrap test with 1000 replicates was performed to ensure confidence in phylogeny. A median-joining haplotype network was also generated based on the aligned sequences using SplitsTree 4.13.1 (Huson and Bryant, 2006). Motif change patterns due to SNPs in *Wx* promoter region and intron 1 of Bangladeshi cultivars were predicted using the PLACE database (www.dna.affrc.go.jp/PLACE). Amino acid changes due to SNP in sequenced *Wx* coding exons from selected Bangladeshi landraces were predicted using the ExPASy translation tool (<http://web.expasy.org/translate/>). SplicePredictor (<http://bioservices.usd.edu/splicepredictor/>) was used to predict splice-site modifying SNPs in intronic regions of sequenced varieties. RegRNA (<http://regrna.mbc.nctu.edu.tw/>) was used to predict *cis*-regulatory elements involved in mRNA splicing or transcriptional regulation.

2.6. Semi-quantitative RT-PCR and gene expression

Total RNAs from rice endosperm at 16-18 days after flowering were extracted using TRIZOL. First strand cDNA were synthesized from total RNA following manufacturer's protocol (Invitrogen, USA). *G3PDH* (glyceraldehyde-3-phosphate dehydrogenase) gene-specific primers (Supplementary Table 1) were used to optimize the cDNA concentration for estimating the *Wx* mRNA level. Nanodrop spectrophotometer was used to measure and optimize the working concentration of the cDNA. *Wx* mRNA level was assessed using cycle-dependent semi-quantitative RT-PCR with the *Wx*RT1 primer pair (Supplementary Table 1) for 30, 32 and 35 cycles. Expression levels of samples were estimated by visual inspection after electrophoresis in a 0.9% agarose gel based on the band intensity of the loading control *G3PDH*. Two sets of primer pairs - *Wx*-RT2 and *Wx*-RT3 (Supplementary Table 1) were used to confirm the G/T SNP at the 5'-splice donor site of *Wx* mRNA of Bangladeshi cultivars. *Wx*-RT2 primer pair was used to determine the splicing efficiency of the first intron in the selected landraces. Nipponbare was used as a control as it contains G/T SNP at intron 1 splice donor site of *Wx* gene (Liu et al., 2009).

3. Results

3.1. Amylose content of Bangladeshi cultivars

In order to identify the molecular basis of glutinous rice in Bangladesh, we first evaluated the amylose content (AC) of 22 Beruin and non-Beruin traditional landraces that are locally considered as glutinous and non-glutinous cultivars respectively. These selected cultivars were sampled from intensive rice cultivation zones in Northeastern and Northwestern regions of Bangladesh that are known to harbour many diverse landraces (Elias et al., 2011). Based on their AC, the selected cultivars were assigned into three major categories - intermediate, low and glutinous/waxy. All non-Beruin Bangladeshi cultivars in this study had intermediate AC ranging from 19.1% to 23.3% (Fig. 1A), confirming that these are indeed non-glutinous. In contrast, only seven out of eleven Beruin varieties, traditionally associated as glutinous or sticky rice, had amylose content within the waxy range (0-5.4%), as shown in Fig. 1B. Among the remaining four Beruin varieties, Kathali Beruin Red and Kalo Beruin belonged to the low AC group (AC 13.7% and 8.2% respectively). The two other cultivars Modhu Beruin Red and Push Beruin (AC 23.4% and 22.6% respectively) had intermediate AC, similar to the Bangladeshi non-glutinous cultivars.

3.2. Nucleotide variation at *Wx* upstream regulatory region of Bangladeshi cultivars

We compared the ~2.7 kb *Wx* upstream noncoding region sequences from selected Bangladeshi cultivars to Olsen and Purugganan (2002) reported progenitor haplotypes F (non-glutinous) and G (glutinous). These progenitor haplotypes differ only in the intron 1 G/T splice donor site mutation (Olsen and Purugganan, 2002). Overall, Bangladeshi cultivars had 29 single nucleotide polymorphisms (SNP) and 11 insertions/deletions (indel) in the sequenced upstream regulatory region (Fig. 1C). Out of these, 15 SNPs and 3 indels were found in the promoter regions and 13 SNPs and 7 indels in intron 1. These SNPs and indels in promoter and intron 1 were more frequent in non-glutinous cultivars than the Beruin cultivars. In total, seven different microsatellite alleles: CT₈, CT₁₀, CT₁₁, CT₁₂, CT₁₆, CT₁₇ and CT₁₈, were identified in the *Wx* exon 1 (encoding 5' untranslated region) of Bangladeshi cultivars (Figure 1C). An additional SNP within *Wx* exon 2 that also codes for 5' UTR was observed only in one of the non-glutinous Bangladeshi cultivars.

All of the Bangladeshi non-glutinous cultivars in this study had G-SNP at the 5' splice donor site of *Wx* intron 1 (Fig. 1C) similar to the known non-glutinous haplotype F. The CT₁₀ or CT₁₁ alleles within *Wx* exon 1 were observed in six of these non-glutinous Bangladeshi cultivars. The CT₁₇ allele was present in cultivars with variable AC, ranging from intermediate AC in Bangladeshi non-glutinous cultivars to low and waxy AC content in the Beruin cultivars (Fig. 1). The CT₁₂/CT₁₈ and CT₈/CT₁₆ alleles were specific to non-glutinous and glutinous Bangladeshi cultivars, respectively.

G/T SNP at the 5' splice site of *Wx* intron 1 could explain the waxy or low AC phenotype (AC 4.4-8.2%) of six out of eleven Bangladeshi Beruin cultivars. These cultivars also carried CT₁₇ allele in exon 1 similar to the known glutinous progenitor haplotype G (Fig. 1C). Among these six Beruin cultivars, Pak Beruin, Kathali Beruin white and Kalo Beruin had an additional novel G/A SNP at position 718 in the *Wx* promoter region (Fig. 1C). The remaining five Beruin cultivars lacked the G/T SNP in intron 1 splice donor site. Among these, Push Beruin and Modhu Beruin Red also showed intermediate amylose phenotype (AC 22.6% and AC 23.4% respectively) like non-glutinous Bangladeshi cultivars in this study (Fig. 1A). These varieties also had other nucleotide variations in *Wx* regulatory region that were similar to the Bangladeshi non-glutinous cultivars (Fig. 1C). This confirmed that Push Beruin and Modhu Beruin Red might be locally misnamed as glutinous varieties even though they have non-glutinous phenotype.

The other three Beruin cultivars that lacked the G/T SNP in splice donor site (Khara Beruin, Modhu Beruin White and Kathali Beruin Red) had waxy or low AC phenotype (Fig. 1B). Among these only Khara Beruin showed several unique mutations in the *Wx* upstream noncoding region (T/A SNP at 216 position in promoter, G/T SNP at position 1934 in intron 1). This indicated that polymorphisms other than G/T SNP in intron 1 splice donor site and CT_n allele in exon 1 may play a major role in regulating *Wx* gene expression in these three genotypes.

3.3. Evolutionary relatedness of Bangladeshi cultivars

An unrooted phylogenetic tree based on the aligned ~2.7kb *Wx* upstream regulatory region sequences

of selected Bangladeshi cultivars and 18 other reported haplotypes (Olsen and Purugganan, 2002) was constructed to explore the evolutionary relationships among these accessions. These 18 reported haplotypes represent the landrace diversity across Asia. In the phylogenetic tree, six Bangladeshi glutinous cultivars (Akia Beruin White, Akia Beruin Red, Mou Beruin, Kalo Beruin, Kathali Beruin White and Pak Beruin) clustered with reported glutinous haplotypes G, I, K, L and Q at high bootstrap (98%) value (Fig.2). Pak Beruin, Kalo Beruin, Kathali Beruin White along with a non-glutinous cultivar Shamudrophena also formed a sub clade depending on the G/A SNP in position 718 of the *Wx* promoter region (Fig.2). These cultivars may carry the same *Wx* allele or with an additional mutation (718 G/A).

On the other hand, five glutinous Beruin cultivars that lacked the intron 1 G/T SNP were grouped with reported non-glutinous haplotypes and Bangladeshi non-glutinous cultivars. Intermediate AC cultivars Push Beruin and Modhu Beruin Red clustered with reported non-glutinous haplotypes B, A and Bangladeshi non-glutinous cultivars (Fig.2). Waxy cultivar Khara Beruin clustered with reported non-glutinous haplotypes E and D at 64% bootstrap and with non-glutinous haplotype C at 93% bootstrap (Fig.2). Based on the aligned *Wx* upstream regulatory region sequences, waxy AC Modhu Beruin White and low AC Kathali Beruin Red appeared to be very close to intragenic recombinant haplotype R and non-glutinous haplotype S, respectively, even though neither shared similar amylose phenotype with the corresponding haplotypes (Fig.2). Bangladeshi non-glutinous cultivars were clustered with reported non-glutinous haplotypes but were more close to the haplotype F, A and B (Fig.2). In addition, Chinigura, Kalijira and Kataribhog distinctly clustered with non-glutinous progenitor haplotype F at 48% bootstrap (Fig.2). It might be possible that the Bangladeshi non-glutinous cultivars originally carried the non-glutinous progenitor *Waxy* allele and then evolved into haplotypes A and B through subsequent mutations at promoter region and intron 1.

3.4. Nucleotide variations at coding region of *Wx* gene in selected Beruin cultivars

Since G/T SNP at intron 1 splice donor site was not enough to explain the waxy AC of Modhu Beruin White and Khara Beruin and low AC of Kathali Beruin Red, we further sequenced two segments in

the coding region of *Wx* gene. Mou Beruin (AC 4.4%) and Shamudrophena (AC 23.4%) were considered as references for Bangladeshi glutinous and non-glutinous cultivars respectively for this analysis. The first sequenced segment in *Wx* gene included part of coding region of exon 2 (~196 bp) which has been reported to contain a 23bp frame-shift duplication in some glutinous varieties that lead to non-functional *Wx* proteins (Mikami et al., 2008; Wanchana et al., 2003). Among the selected cultivars, this 23bp duplication was only identified in Mou Beruin, consistent with its glutinous phenotype (Table 1). The second segment analyzed in *Wx* gene spanned ~1.9 kb genomic region including 3' end of exons 2-10 and introns 2-10. Only the non-glutinous reference Shamudrophena among the five cultivars carried A/C SNP in exon 6 (position 671 from start codon), which resulted in nonsynonymous amino acid change from tyrosine to serine (Table 1). This SNP has been shown to be associated with intermediate AC (21-24%) previously (Dobo et al., 2010). Two other coding region SNPs (T/C in exon 9, C/T in exon 10) were identified in the three selected Beruin cultivars but not in the non-glutinous or glutinous reference (Table 1). Exon 9 SNP (T/C, position 1109 from start codon) was found in all three selected cultivars (Table 1). The exon 9 SNP results in a synonymous change in amino acid and has been previously reported as a silent mutation (Larkin and Park, 2003). Exon 10 SNP (C/T) was found only in low AC Kathali Beruin Red (Table 1). Exon 10 SNP (C/T) caused a nonsynonymous change from a nonpolar amino acid proline (CCT) to polar amino acid Serine (TCT), and it has been reported that this SNP is linked to high amylose content (Larkin and Park, 2003). Therefore these exon-specific mutations were not directly responsible for the variant AC phenotype traits observed for the selected Beruin cultivars.

Most interestingly, Kathali Beruin Red, Modhu Beruin White and Mou Beruin had overall four SNPs and two indels in *Wx* introns which were absent in the Bangladeshi non-glutinous and glutinous references (Table 1). Among these variations, C deletion in intron 5, G/A (at positions 81 and 95 from 5' splice site of intron 10) and ATA deletion at position 104-106 in intron 10 were identified in all these three cultivars. On the other hand, G/A in intron 10 (position 118) was found only in low AC Kathali Beruin Red and A/G at position 29 from 5' splice site of intron 9 was only observed in the waxy ACKhara Beruin and Modhu Beruin White. A search for putative cis-elements that might be

affected by these SNPs/indel revealed that the C indel was located within a poly-C tract in intron 5, and this indel resulted in absence of a putative INTRONLOWER motif in these three cultivars (Supplementary Table 2). Also, the G/A indel in intron 10 (at position 95 from 5' splice site) resulted in a missing putative ESE-SF2/ASF motif (Exonic Splicing Enhancer) in these three cultivars (Supplementary Table 2).

Further homology search using NCBI BLASTn revealed that the sequenced coding regions of Khara Beruin and Modhu Beruin White shared ~99% identity with previously reported Wx^{op} (opaque) allele containing cultivars ARC10818, ACC35618 and ARC6622 (Genbank accessions AB281448.1, AB281447.1, and AB281453.1) from Nepal and India (Mikami et al., 2008, 1999). These Wx^{op} accessions did not contain the G/T SNP at the 5' splice donor site of intron 1 and also had nonsynonymous A/G SNP in exon 4 at position 521 from start codon (Mikami et al., 2008, 1999). However the latter exon 4 SNP was not present in the Khara Beruin and Modhu Beruin White (Table 1). Comparison of the rice grains revealed that only Khara Beruin had a completely opaque or chalky endosperm, while Modhu Beruin White had partially chalky endosperm (Fig. 3), which supports the low to very low amylose content of these varieties.

3.5. Wx gene expression in selected Beruin cultivars without G/T SNP in splice donor site

Semi-quantitative RT-PCR was used to estimate total expression levels of Wx gene in Modhu Beruin White (waxy AC), Kathali Beruin Red (low AC), Push Beruin and Modhu Beruin Red (intermediate AC). Nipponbare was used as a control since it is known to show decreased splicing efficiency of Wx transcript due to G/T SNP at intron 1 splice donor site (Liu et al., 2009). The results showed that Wx transcript levels in Push Beruin, Modhu Beruin Red and Kathali Beruin Red were relatively much higher compared to the control Nipponbare, (Fig. 4A). This suggests that the splicing efficiency of Wx transcript is not affected in Push Beruin, Modhu Beruin Red as well as Kathali Beruin Red. This observation is consistent with the absence of G/T SNP at intron 1 and the intermediate-to-low AC in these cultivars. The expression level of Wx transcript in Modhu Beruin White (intron 1 splice site mutation absent) was also higher than that of Nipponbare but lower than the aforementioned three

Beruin cultivars (Fig.4A).The transcript level however correlates with the waxy AC of Modhu Beruin White.

RT-PCR with primers specific for unspliced variant of *Wx* transcript (Liu et al., 2009) confirmed the absence of intron 1 containing 1.1 kb fragment in all of the selected Beruin cultivars (Fig. 4B). A second set of RT-PCR primers previously described by Prathepha (2007) was used to re-confirm the absence of G/T SNP at intron1 splice donor site of *Wx* gene of these selected cultivars (Fig. 4C). Nipponbare showed a predominant band at 120 bp and another band at 210bp, while the selected Beruin cultivars only showed the 210 bp band (Fig. 4C). This is consistent with previous observations that cultivars carrying intron 1 G/T SNP showed a predominant band at 120 bp with an additional band at 210bp, indicating at least two alternative variants of *Wx* transcript, but cultivars without the mutation showed only a predominant band at 210bp (Prathepha, 2007).

4. Discussion

4.1. Distribution of Bangladeshi rice cultivars in context with Asian rice cultivars

In this study, 22 Bangladeshi glutinous and non-glutinous cultivars were placed in specific positions along with 18 previously reported *Wx* haplotypes (Olsen and Purugganan, 2002) based on phylogenetic- and haplotype network analyses of *Wx* upstream regulatory region sequences (Fig. 2 and 5). The previously reported *Wx* haplotypes were identified using 105 accessions from different countries of Asia (Olsen and Purugganan, 2002). Bangladeshi non-glutinous cultivars Kalijira, Kataribhog and Chinigura have the same *Wx* allele as haplotype F with no additional mutation. As such, these cultivars were placed with non-glutinous progenitor haplotype F. Previously, Olsen and Purugganan (2002) also showed that 39% of accessions in Southeast Asia and 15% of the South Asian accessions contain this F haplotype. Four non-glutinous cultivars (Lathial White, Lathial Red, Girishail, and Raujan2) and one intermediate amylose containing cultivar Modhu Beruin Red bear closer identity to haplotype A and were placed with it (Fig. 5).

Three other Bangladeshi non-glutinous cultivars - Balam White, Balam Red and Raujan, and one intermediate amylose containing cultivar Push Beruin were placed in the same node as haplotype B (Fig.5). Non-glutinous haplotypes A and B were derived from haplotype F by subsequent mutations at both promoter and intron1 region. Haplotype A is more frequent in Southeast Asia and haplotype B is more frequent in South Asia(Olsen and Purugganan, 2002). Therefore, Bangladeshi non-glutinous and Beruin rice cultivars with intermediate AC carry the general pattern prevalent in South and Southeast Asia.

Among the Bangladeshi glutinous cultivars, Mou Beruin, Akia Beruin White and Akia Beruin Red have the same sequence identities as the glutinous progenitor haplotype G in *Wx* upstream region (Fig.5). Most of the glutinous rice accessions in Southeast, North and South Asia have been reported to carry haplotype G. Bangladeshi glutinous cultivars Kalo Beruin, Pak Beruin and Kathali Beruin White contained an additional G/A SNP at position 718 of *Wx* promoter along with the G/T SNP at the 5' splice donor site of intron 1 (Fig. 1). These cultivars clustered with the glutinous haplotype group (L, K, O and I), which also have additional mutations located at promoter and intron 1 but at different positions (Fig.5). The 718 (G/A) SNP has not yet been reported and is therefore unique to Bangladeshi glutinous cultivars as well as the non-glutinous Shamudrophena.

From the median-joining network analysis (Fig.5), low AC Bangladeshi Kathali Beruin Red was found to be close to the non-glutinous haplotype S. Two other waxy AC cultivars Khara Beruin and Modhu Beruin White were placed with non-glutinous haplotype C and recombinant haplotype R in the *Wx* haplotype network (Fig.5). This suggests that some Bangladeshi glutinous Beruin cultivars may have a different allele compared to glutinous cultivars of Asia responsible for waxy or low AC phenotype.

4.2. Bangladesh genotype-specific changes

Several studies have reported specific SNP or indels in *Wx* exon that decrease AC in rice seeds by reducing the binding of GBSS-I to starch granules or creating stop codon that terminates the translation of *Wx* transcript (Liu et al., 2009; Wanchana et al., 2003). However, waxy Khara Beruin,

Modhu Beruin White and Kathali Beruin did not have any of those SNPs. Pairwise sequence comparisons of these cultivars with Olsen and Purugganan (2002) reported haplotypes showed that some mutations at both promoter and intron 1 region were shared between Khara Beruin and haplotype C (99.93% sequence identity), Modhu Beruin White and haplotype R (99.19% identity) as well as Kathali Beruin Red and haplotype S (99.26% identity). Some of these mutations were found to be located within putative cis-regulatory motifs in the *Wx* promoter site in Beruin cultivars (Supplementary Table 2). Most interestingly, these three cultivars have a C deletion at a polypyrimidine tract of intron 5 as well as two G/A SNPs and an ATA deletion at intron 10. This C deletion in intron 5 consequentially causes loss of the putative INTRON LOWER motif in that region. This may cause slippage of intron splicing and subsequent translation (Brown, 1986; Sridharan and Singh, 2007). Also, the G/A indel in intron 10 (at position 95 from 5' splice site) which affected a putative Exonic Splicing Enhancer motif might cause exon skipping in these three cultivars (Supplementary Table 2). Therefore, these mutations might be another cause of the low AC in Khara Beruin, Modhu Beruin White and Kathali Beruin Red despite lack of the G/T SNP at the intron 1 splice donor site, which is known as the main cause of waxy or low amylose phenotype.

Waxy amylose containing Khara Beruin and Modhu Beruin White also contained one SNP at intron 9 (G/A) and showed 99% similarity with opaque or chalky endosperm containing rice cultivars found in Nepal, India, Myanmar and Indonesia. On the other hand, low amylose containing Kathali Beruin Red contained one G/A SNP in position 118 in intron 10 that might create an alternative splice acceptor site in intron 10 (Supplementary Table 2). This SNP in intron 10 has not been reported previously. These SNPs at introns 9 and 10 also might have an effect on splicing as well as gene expression and varying amylose between waxy Khara Beruin, Modhu Beruin and Low AC Kathali Beruin Red. However, the transcript levels of the *Wx* gene in these three Beruin cultivars were not low compared to Nipponbare, which carries the G/T splice site SNP in intron 1. Whether the above SNPs in the promoter and introns alone are responsible for regulating *Wx* transcript levels in these cultivars or other players are involved requires further investigation.

5. Conclusion

Bangladeshi non-glutinous and glutinous cultivars with intermediate AC carried the general pattern of nucleotide variation in *Wx* upstream region as observed in South and Southeast Asian cultivars. Some exceptional *Wx* alleles were also identified in Bangladeshi glutinous cultivars Khara Beruin, Modhu Beruin White and Kathali Beruin Red. These cultivars lack the G/T splice site mutation in intron 1 region as non-glutinous cultivars, but yet have waxy and/or low amylose phenotype. However these cultivars have some SNPs in promoter region that may alter putative cis-regulatory motifs involved in *Wx* gene regulation. Additionally, a putative INTRON LOWER motif (consensus sequence for plant introns) and an Exonic Splicing Enhancer motif were lost in these cultivars due to indels in *Wx* introns 5 and 10 respectively. Khara Beruin and Modhu Beruin White (waxy AC) also contained a SNP at intron 9 and Kathali Beruin Red (low AC) contained two SNPs at introns 6 and 10. It can be hypothesized that the intron- and promoter-specific mutations found in these three cultivars could be the cause of the waxy or low amylose phenotype. This hypothesis however needs to be investigated further through functional characterization of these motifs and confirmation of alternative *Wx* variants due to misregulation of splicing.

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