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RESEARCH ARTICLE

Germplasm Characterization

Assessment of submergence stress responses and mining allelic variations of submergence tolerance gene *Sub1A* in Sri Lankan rice germplasm[1]

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Abstract: The development of submergence tolerant (ST) rice cultivars is a priority in rice breeding programmes globally, as floods significantly curtail rice production. *Sub1A* is the major known gene conveying submergence tolerance in rice, carrying two variants; *Sub1A-1* (tolerance allele) and *Sub1A-2* (susceptible allele). Sri Lanka hosts genetically diverse rice germplasm; however, the allelic diversity of the *Sub1A* gene among the Sri Lankan rice germplasm remains unknown. Hence, the current study focuses on the assessment of submergence stress responses and identification of the allelic diversity of the *Sub1A* gene in a panel of 64 Sri Lankan rice varieties/accessions. Submergence stress responses of the rice panel were evaluated and *Sub1A* allelic variations were assessed using a modified gel-based marker, *ABUOP0003*. A Bayesian approach was used to assess the genetic structure of the rice panel and its association with the *Sub1A* alleles was explored. Thirteen rice accessions/varieties showed significantly similar ($p > 0.05$) survival percentages (SP - 77 to 93%) as ST reference FR13A. The *ABUOP0003* marker assay revealed that 13 rice accessions/varieties carry *Sub1A-1*. The rice accessions *Godaheenati, Heenkarayal, Goda wee* and *Dik wee* carried the *Sub1A-1* allele, as well as expressing a tolerance response to submergence stress. No significant association ($p < 0.05$) was observed between the *Sub1A* alleles and submergence stress responses indicating additional genes at play. The deduced genetic structure of the rice panel revealed two genetically isolated gene pools, and the *Sub1A* alleles did not associate with any specific gene pool, indicating that the allele divergence pre-dates germplasm introductions and varietal development attempts in Sri Lanka**.**

Keywords: Marker *ABUOP0003*, rice, Sri Lankan rice germplasm, *Sub1A-1* allele, submergence tolerance.

INTRODUCTION

Among the widely cultivated crops, rice (*Oryza sativa* L.), despite its semi-aquatic habit, is one of the most flood-prone crops. During rainy seasons paddy fields are subjected to frequent flooding due to poor drainage, which results in partial or complete inundation for prolonged periods. This limits the supply of oxygen and sunlight to rice plants, hindering photosynthesis and leading to a shortage of overall cellular energy (Loreti *et al*, 2016). It is reported that more than 15% of the global paddy fields are affected by floods annually, causing significant harvest losses (Bailey-Serres & Voesenek, 2008).

Rice can be subjected to submergence stress at any growth stage. Complete submergence at the germination stage could result in poor germination, developmental delays, and seedling death due to anoxia or hypoxia stress conditions (Ismail *et al.*, 2009; Loreti *et al*., 2016). However, rice plants carrying genetic tolerance could germinate even under such conditions, due to adaptations such as rapid coleoptile elongation and delayed radicle development (Ismail *et al.*, 2009; Magneschi & Perata, 2009; Hattori *et al.*, 2011). During the vegetative phase (seedling stage or tillering stage), submergence tolerant plants can withstand inundation in

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flood waters for 10-14 days. As an adaptation to withstand submergence stress the tolerant plants either maintain a suppressed growth (up to an inundation period of 2 weeks; Bailey-Serres *et al.*, 2010) or increase their height (up to an inundation period of 4 weeks; Hattori *et al.*, 2011). Even though most tolerant rice plants will resume their growth with the receding flood waters, some plants will eventually die due to lodging and exhaustion of energy reserves (Winkel *et al.*, 2013).

Tolerance to submergence is genetically controlled by quantitative trait loci (QTL). Among them, *Sub1* is the major QTL involved with submergence tolerance in rice, and it is mapped to the rice chromosome 9 (Xu & Mackill, 1996). The *Sub1* is the main QTL conveying submergence tolerance and it accounts for 69% of the phenotypic variance in rice submergence tolerance (Xu & Mackill, 1996). The *Sub1* consists of three main genes, *Sub1A*, *Sub1B,* and *Sub1C* (Xu *et al.*, 2006). Of the three genes, *Sub1A* is the major contributor towards submergence tolerance. The *Sub1A* gene is known to be bi-allelic (*Sub1A-1* and *Sub1A-2*), where *Sub1A-1* is known to convey a tolerance response and *Sub1A-2* a susceptible response to submergence stress (Xu *et al.*, 2006). According to Xu *et al.* (2006), the *Sub1A* gene is absent in *japonica* rice varieties and the two alleles of *Sub1A* are reported in some *indica* rice varieties. Identification of rice accessions/varieties carrying the tolerance *Sub1A-1* allele will give the rice breeders the ultimate advantage of incorporating the best genetic background against submergence stress in released rice varieties. In previous studies, Sri Lankan traditional rice accessions *Kurkaruppan*, *Godaheenati,* and *Thavalu* have been identified to carry the *Sub1A-1* tolerance allele (Vergara & Mazaredo, 1975; Xu & Mackill, 1996; Xu *et al.*, 2006; Bailey-Serres *et al.*, 2010; Panda *et al.*, 2021). However, the *Sub1A* alleles carried by most Sri Lankan rice accessions/varieties remain unknown. The availability of this vital genomic information is beneficial for rice breeders to make informed decisions in rice breeding programmes. Therefore, in the current study, submergence tolerance responses of 64 Sri Lankan rice accessions/varieties, along with two known submergence stress response references FR13A for tolerance and *Swarna* for susceptibility to submergence (Xu *et al.*, 2006; Neeraja *et al.*, 2007; Singh *et al.*, 2010; Emerick & Ronald, 2019; Baksh *et al.*, 2021) were assessed in a replicated field trial in artificial screening tanks. Further, the *Sub1A* alleles carried by the respective rice accession/variety were identified using a modified gel-based molecular marker, to identify and recommend potential donors carrying *Sub1A-1* mediated genetic tolerance to submergence for the use in rice breeding programmes.

MATERIALS AND METHODS

Plant material

For the current study, a panel of 66 rice accessions consisting of 40 Sri Lankan newly improved rice varieties (SLNIV), 24 Sri Lankan traditional rice accessions (SLTA), and two references (submergence tolerant reference line FR13A and submergence susceptible reference variety *Swarna*) were used (Supplementary file S1). Rice seeds were sourced from the Plant Genetic Resources Centre, Gannoruwa, Sri Lanka and the Regional Rice Research and Development Centre, Bombuwala, Sri Lanka. DNA extraction of these rice accessions/varieties was carried out using a modified CTAB method (Doyle & Doyle, 1987).

Genetic structure and diversity analysis

Sixty-four rice accessions consisting of SLNIVs and SLTAs were subjected to PCR amplification using 12 unlinked molecular markers representing all rice chromosomes (Supplementary file S2). A PCR amplification was carried out in a thermal cycler (CT1000, Bio-Rad, USA) using a 15 µL final PCR reaction volume with 150 ng DNA template, 1× Gotaq® Green Master Mix (Promega Corporation, Madison, Wisconsin, USA), 0.33 µM forward and reverse primers each, volumed up with nuclease free water (Promega Corporation, Madison, Wisconsin, USA). The PCR amplicons were resolved and visualized on a 3% (w/v) Agarose gel (Sigma Aldrich, Merck group, Germany) pre-stained with 5% (v/v) ethidium bromide (Sigma Aldrich, Merck group, Germany) using a gel documentation system (UVCI-1100, Major Science, USA). The rice accessions/varieties were genotyped based on the resulting band sizes defined based on a 100 bp DNA ladder (Promega Corporation, Madison, Wisconsin, USA).

Bayesian structure analysis was performed using an admixture model in Structure v2.3.4 (Pritchard *et al*., 2000). To resolve the optimum most probable number of ancestral clusters (K), the score matrix was analyzed

assuming K = 1 to K = 10 clusters with repetitive ten runs for each K, 500,000 Markov Chain Monte Carlo iterations (MCMC), and a burn-in period of 200,000 iterations. The optimum K was derived based on log-likelihood values in Structure Harvester v0.6.93 (Earl & vonHoldt, 2012) based on the delta-K against K Evanno plot. The optimum K value was used with 700,000 MCMC iterations and a burn-in period of 300,000 iterations for the final run in Structure v2.3.4. The resulting outcome was modified using Structure Plot v2.0 (http://omicsspeaks.com/strplot2/) and the resulting plot and the probability values were examined to deduce the possible historical genetic admixtures present within the rice panel considered.

Allelic characterization of rice panel for *Sub1A* using *ABUOP0003* marker

The *Sub1A-1* genomic sequences of the rice breeding line IR40931 (a descendant of the submergence tolerant breeding line FR13A) were retrieved from the National Centre for Biotechnology Information (NCBI) repository (GenBank accession number: DQ011598; Xu *et al.*, 2006), and *Sub1A-2* genomic sequence of the Cultivar 93-11 (a submergence susceptible cultivar) was retrieved from the Gramene Database (Gene ID: BGIOSGA038325; Ganie *et al.*, 2020). The sequences were aligned using the ClustalW feature (Thompson *et al.*, 2003) in Geneious v.7.1.3 (Kearse *et al.*, 2012), and the MAPK (mitogen-activated protein kinases) site carrying the T/C SNP assayed by the previously published dominant sequence tagged site (STS) marker *AEX1* (Septiningsih *et al.*, 2009) was annotated. Based on Septiningsih *et al.* (2009), the sequences of the *AEX1* forward (*AEXF*; common primer hereinafter named as *ABUOP0003F*; 5'-AGGCGGAGCTACGAGTACCA-3'sequence) and allele specific primer (*AEX1R*; T allele specific primer at MAPK site hereinafter named as *ABUOP0003R1*; 5'-GCAGAGCGGCTGCGA-3' sequence) were retrieved and annotated on to the *Sub1A* sequence alignment of IR40931 and Cultivar 93-11. To convert the dominant *AEX* marker to a co-dominant state, a second allele specific primer was designed targeting the alternative allele at the MAPK site (C allele specific primer referred hereinafter as *ABUOP0003R2*; 5'-GCAGAGCGGCTGCGG-3' sequence) using the Primer3 (Untergasser *et al.*, 2012) feature in Geneious v7.1.3 (Kearse *et al.*, 2012).

The two primer pairs *ABUOP0003F/R1* and *ABUOP0003F/R2* were separately assayed on three rice accessions (FR13A, known to carry *Sub1A-1*; Cultivar 93-11, known to carry *Sub1A*-2; and *Swarna*, known not to carry the *Sub1A* gene) and an artificial heterozygote (created by mixing equal quantities of DNA of FR13A and Cultivar 93-11). The PCR amplification of *ABUOP0003* was carried out for the DNA samples using a 15 µL final PCR reaction volume containing 150 ng template DNA, 1× of GoTaq® Green Master Mix (Promega, USA), 0.33 µM concentration of the primer combination *ABUOP0003F/R1* and 0.26 µM of *ABUOP0003F/R2* in a thermal cycler (CT1000, Bio-Rad, USA) with 1 mg/mL Bovine Serum Albumin. For the non-template control sample, template DNA was replaced by nuclease-free water (Promega, USA). The touchdown PCR programme consisted of the following steps: an initial denaturation step at 95 ºC for 5 min followed by 10 cycles of PCR amplification with 30 s denaturation step at 95 ºC, 30 s primer annealing touchdown step at 65 ºC with a drop of 0.5 ºC per each cycle and 45 s primer extension step at 72 ºC. This was followed by 30 cycles of 30 s denaturation step at 95 ºC, 30 s primer annealing at 60 ºC and 45 s primer extension step at 72 ºC and a final primer extension step for 5 min at 72 ºC. The amplified products were resolved and visualized on a pre-stained 3% (w/v) agarose gel with 5% (v/v) ethidium bromide. In order to resolve the same sized allele-specific products resulted from the separate-tube PCR (carried out with primer combinations of *ABUOP0003R1/F* and *ABUOP0003R2/F*) as a co-dominant assay, the resultant PCR products of the same DNA samples were loaded sequentially with a 10 min interval apart. The gel image was captured using a gel documentation system (UVCI-1100, Major Science, USA) and the *Sub1A* alleles were scored as *Sub1A-1* 'tolerant allele', *Sub1A-2* 'susceptible allele' or as heterozygous in the tested rice panel. In the event of repeated no amplification, the reason was thought of as absence of the *Sub1A* gene or a PCR failure. The *ABUOP0003* assay was repeated for the remaining accessions/varieties of the core rice panel and the respective alleles carried by them were determined.

Field evaluation for submergence stress responses

Field evaluation of the 66 rice accessions/varieties for submergence stress responses was conducted at the Regional Rice Research and Development Centre, Bombuwala, Sri Lanka, following methods described by Singh *et al.* (2010) and Emerick & Ronald (2019) in artificial screening tanks. Healthy rice seeds from each rice accession/variety were sown onto paddy soil-filled troughs, following a completely randomized design layout in

three replicates. After 2 wks, in each replicate, the additional seedlings were thinned out leaving ten healthy seedlings with similar physical appearance. On the 14th day after sowing, the number of surviving seedlings was counted and the seedling troughs intended for submergence stress treatment were placed in concrete tanks filled with irrigated water to a 1 m depth for a duration of 2 wks. The troughs intended to serve as the control experiment were maintained in a separate tank without submerging and were kept well-watered. At the 14th day after submergence, the seedling troughs in the submergence stress treatment were de-submerged for a period of 14 ds and the survival percentage of each rice accession/variety was calculated based on the number of surviving seedlings. The survival rates of the control experiment were used to check for germination and general survival of seedlings during the experimentation period, where above 80% survival at the end of 21 ds after sowing was considered as the acceptable standard. For the treatment experiment, the estimated median of the survival percentage for each accession/variety was calculated using the Friedman test in Minitab 17 (Minitab, Inc.; www.minitab.com). Based on the estimated median scores, the rice accessions/varieties, which showed no significant difference ($p > 0.05$) to FR13A, the known submergence tolerant line, were grouped as 'tolerant'. Further, the rice accessions/varieties which showed no significant difference ($p > 0.05$) to *Swarna*, the known submergence susceptible variety, were grouped as 'susceptible' and the rice accessions/varieties, which are significantly more susceptible ($p > 0.05$) than *Swarna,* were grouped as 'highly susceptible'. The accessions/varieties categorized in-between were considered as the 'intermediate' group. A pairwise comparison was carried out using the Mann-Whitney U test, and a Box and Whiskers plot was constructed in Minitab 17 (Minitab, Inc.; www.minitab.com) to visualize the survival percentage of the accessions/varieties carrying *Sub1A-1* and *Sub1A-2* under submergence stress.

RESULTS AND DISCUSSION

Changes in the global climate have made rainfall patterns unpredictable leading to frequent inundation of lowland rain fed paddy fields for prolonged periods causing submergence stress in rice plants. This has led to a significant decrease in the productivity of these lands and the situation is worsening as most rice varieties in cultivation are not suitable for such flood-prone areas. Therefore, with limited options to prevent flooding from devastating the croplands, exploration of genetic potential in rice to survive prolonged submergence and recovery is an important aspect that has caught the attention of the rice breeders globally. Sri Lanka is host to many rice accessions, where the genetic potential of some Sri Lankan rice germplasm (SLNIVs and SLTAs) in terms of tolerance to submergence has been reported in several studies (Ranawake *et al.*, 2013; Ranawake *et al.*, 2014) and in varietal descriptors. However, the submergence response of many other accessions, especially some of the SLTAs remain unknown.

Submergence tolerance in rice is controlled by the *Sub1* gene cluster, with major tolerance conveyed *via Sub1A* gene. However, the alleles of *Sub1A* carried by most Sri Lankan rice accessions/varieties remain unknown to date. In the current study, FR13A (the tolerance reference) and *Swarna* (the susceptible reference) were used to identify submergence tolerant and susceptible rice accessions/varieties from a core rice panel consisting of 64 rice accessions/varieties (40 SLNIVs and 24 SLTAs) in a replicated field trial conducted in artificial screening tanks and *Sub1A* alleles carried by these rice accessions/varieties were identified using a modified gel-based DNA marker for the use in rice breeding programmes.

Genetic structure and diversity analysis of the core rice panel

To understand the genetic structure of the core rice panel used in the current study, a Bayesian genetic assessment analysis was conducted. Based on the Structure Harvester Evanno plot, the optimum most probable number of ancestral clusters (K; Figure 1A) for the study panel was set at K = 2 (Figure 1B - Group 1 (green) and Group 2 (brown)). Group 1 consisted of 39 assigned genotypes, all belonging to SLNIVs, and Group 2 consisted of 18 assigned genotypes belonging to SLTAs (≥ 95% assignment probability). The remaining seven accessions/varieties were grouped as carrying historical genetic admixtures, with *Murungakayan, Maa wee* and Bw 272-6b resembling mostly Group 1 (with an assignment probability 64-77%), and *Pachchaperumal, Pokkali, Dahanala* and *Dik wee* resembling mostly Group 2 (with an assignment probability of 88-94%). Further, in the core rice panel, 97.5% of the SLNIVs were assigned to Group 1 and 75% of the SLTAs were assigned to Group 2. Hence in general SLNIVs and SLTAs are representing genetically isolated gene pools at a ≥ 95% assignment probability.

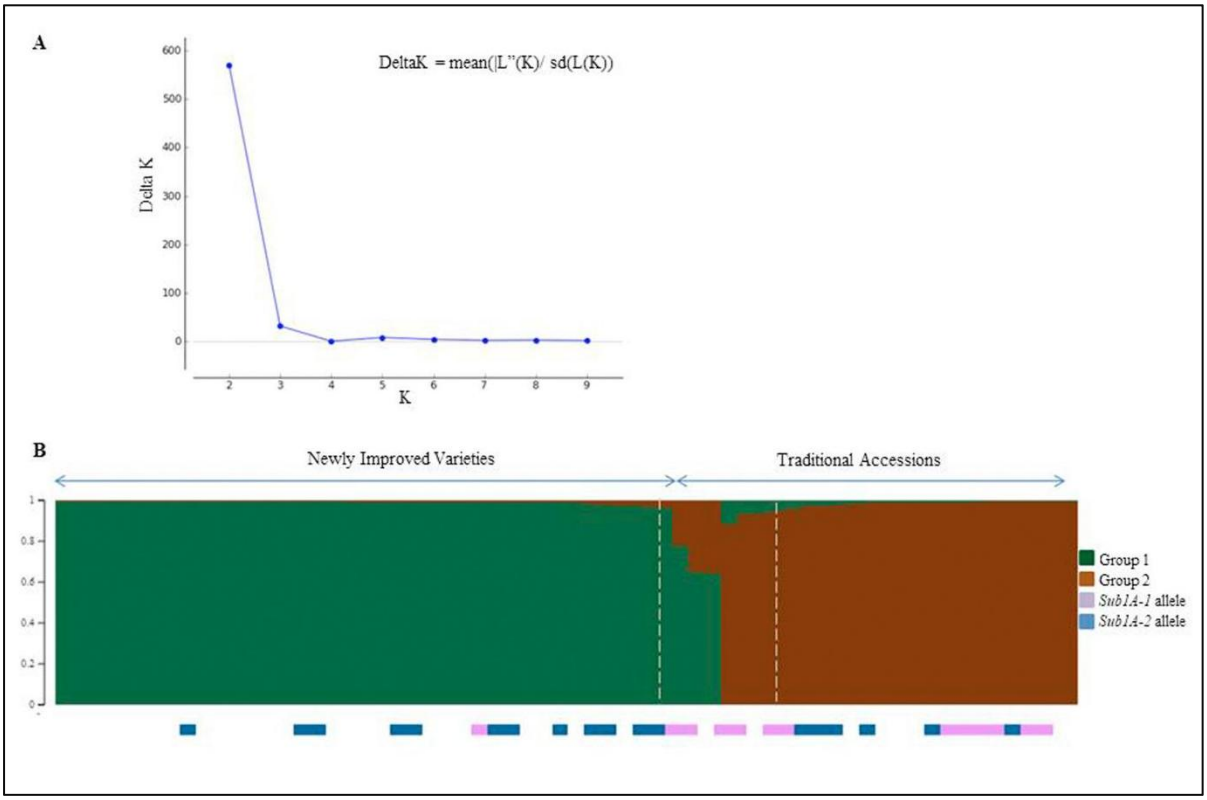

Figure 1: Illustration of (A) Structure Harvester Evanno plot with optimal K and (B) genetic structure of 64 Sri Lankan rice accessions/varieties based 12 genome-wide unlinked DNA markers and the variation of *Sub1A* alleles based on the *ABUOP0003* marker

Characterization of rice accessions for *Sub1A* alleles with *ABUOP0003*

The major gene conveying submergence tolerance in rice is *Sub1A,* and of its two allele forms (*Sub1A-1* and *Sub1A-2*) only *Sub1A-1* conveys tolerance to submergence stress (Xu *et al*., 2006). Therefore, the identification of rice accessions/varieties that carry the tolerance allele *Sub1A-1* is important to make informed decisions at critical decision making junctions in rice breeding programmes. To track the *Sub1A* alleles, the marker *AEX*, which interrogates an SNP at the MAPK site in the *Sub1A* gene, is commonly used. According to Xu *et al.* (2006) and Septiningsih *et al.* (2009), *AEX* is a dominant STS marker designed to amplify the *Sub1A-1* tolerance allele (T of codon TCG; resulting in a serine at the 186 amino acid position in the SUB1A amino acid sequence), but not the *Sub1A-2* susceptible allele (C of codon CCG; resulting a proline at the same amino acid position). However, due to its dominant nature, the marker *AEX* was unable to differentiate heterozygotes and the *Sub1A-2* allele from an absent *Sub1A* gene or a failed PCR reaction. As a result, a secondary site in the *Sub1A* gene was targeted using the cleaved amplified polymorphic sequence (CAPs) marker *GnS2* (Neeraja *et al.*, 2007), where the tolerant allele (A) and the susceptible allele (G) could be differentiated with a subsequent restriction enzyme digestion with *AluI/PvuII* enzymes. Though *GnS2* is a codominant marker, the need to use restriction enzyme digestion makes it expensive and time inefficient. As a result, Moon *et al.* (2019) designed a Kompetetive allele-specific polymorphism (KASP) marker covering both sites targeted by *AEX* and *GnS2* for rapid codominant detection of the *Sub1A* alleles. However, the use of KASP markers is restricted in technology-limited countries and hence, in the current study, a modification to the *AEX* marker assay was introduced to amplify the MAPK site polymorphism as a gel-based codominant marker. The modified gel-based marker is hereinafter presented as *ABUOP0003.* The *ABUOP0003* marker assay comprises of three primers (Figure 2A); forward primer of the marker *AEX* as the common forward primer (*ABUOP0003F*), the tolerance allele specific *AEX* reverse primer (*ABUOP0003R1*) and a newly designed susceptible allele specific primer (*ABUOP0003R2*).

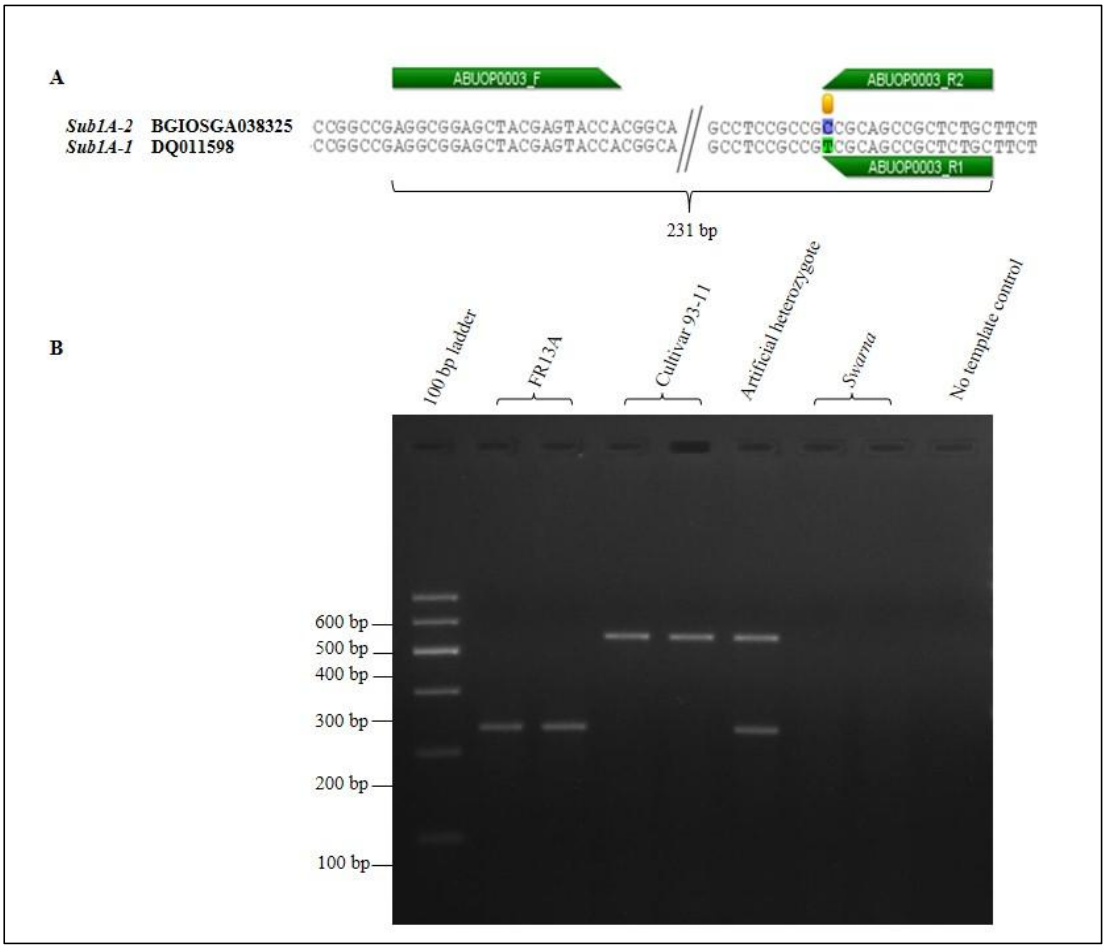

Figure 2: Illustration of the (A) single nucleotide polymorphism and the anchored position of *ABUOP0003* and (B) visualization of the PCR amplicons of *ABUOP0003* for rice accessions *FR13A*, *Cultivar 93-11*, and *Swarna* and an artificial heterozygote.

The primer pairs *ABUOP0003F/R1* and *ABUOP0003F/R2* both amplified a fragment of 231 bp in a separate-tube PCR assay. The primer combination *ABUOP0003F/R1* amplified a fragment only from the submergence tolerant reference FR13A carrying the *Sub1A-1*, and *ABUOP0003F/R2* amplified a fragment only from the susceptible reference Cultivar 93-11 carrying *Sub1A-2* (Figure 2B). Both *ABUOP0003F/R1* and *ABUOP0003F/R2* assays resulted in amplification for the artificial heterozygote. When the PCR products of *ABUOP0003F/R1* and *ABUOP0003F/R2* were loaded onto the same well leading with a 10 min interval on a 1.5 % Agarose gel, the expected polymorphism could be visualized in a co-dominant state (Figure 2B). Further, the marker assay was validated for specificity with the rice accession *Swarna*, known to be without the *Sub1A* gene, where no amplification for marker *ABUOP0003* was observed (Figure 2B). Therefore, the modified gel-based marker *ABUOP0003* was diagnostic to confidently differentiate the alleles *Sub1A-1* from *Sub1A-2*. Therefore, we recommend the modified marker *ABUOP0003,* over the previously used dominant *AEX* STS marker, KASP marker and CAPS marker, as a gel-based, codominant and economical assay, especially targeting technology-limited setups. However, with respect to the absence of a PCR product in *ABUOP0003* assay, it is difficult to confidently rule out a PCR failure from an absence of the *Sub1A* gene. Therefore, it is recommended to assay such accessions/varieties multiple times alongside a positive control.

Out of the panel of 66 rice accessions/varieties, the *ABUOP0003* marker identified 14 rice accessions/varieties as carrying the tolerance allele *Sub1A-1* (FR13A; SLNIVs At 354 and Bw 272-6b; and SLTAs *Maa wee*, *Heen Karayal, Godaheenati, Dik wee, Mada el, Dewareddiri, Pokkali*, *Pachchaperumal*, *Kahata wee*, *Podihatatha* and *Sudugoda wee*). Of these only the accession *Godaheenati* has been previously characterized as carrying the *Sub1A-1* tolerance allele (Xu *et al.*, 2006; Singh *et al.*, 2010). Interestingly, there is no apparent pedigree overlap with respect to these varieties/ accessions (Supplementary file S1). From the tested

rice panel 19 accessions/varieties were identified as carrying susceptible *Sub1A-2* allele, of which 13 were SLNIVs and six were SLTAs (Supplementary file S3). Despite repeated attempts conducted alongside positive controls, the remaining 32 rice accessions (*Swarna*, 25 SLNIVs and seven SLTAs) did not result in PCR amplification for the marker *ABUOP0003* (Supplementary file S3). While there is a possibility that these 32 rice varieties/accessions do not carry the *Sub1A* gene, like in variety *Swarna*, a definitive conclusion could not be drawn despite the repeated attempts to rule out a possible PCR failure. Out of the 64 rice accessions in the core rice panel, 20% carried the *Sub1A-1* tolerance allele and 30% carried the *Sub1A-2* susceptible allele (Supplementary file S3).

The genetic structure and the diversity analysis of the core rice panel indicated minimal evidence of gene flow between the SLNIVs and SLTAs (Figure 1B). According to Pucciariello and Perata (2013), the wild rice accessions from Sri Lanka revealed no evidence of carrying *Sub1A* gene, and they reported several landraces that carried *Sub1A-1*and *Sub1A-2* alleles, indicating the possibility of early settlers bringing in seeds containing *Sub1A* gene from the Indian basin or later introduction of germplasm carrying *Sub1A* through exotic lines used in breeding programmes. The current study further strengthens the theory presented in Pucciariello and Perata (2013), where *Sub1A* alleles were identified from both SLNIVs (*i.e.*, newly developed mainly through introductions) and SLTAs (*i.e.*, including landraces and heirloom accessions) even under the proposed limited gene flow scenario (Figure 1B).

Field evaluation for identification of submergence tolerant rice accessions/varieties

In the current study, phenotypic assessment of submergence responses was assessed in order to deduce the associations of the submergence responses with the allele carried at the *Sub1A* gene. Furthermore, it was intended to identify tolerant rice accessions/varieties that should be recommended as tolerance donors for the development of bi-parental/multi-parental mapping populations for QTL identification and varietal development programs (*i.e.*, through backcross breeding and gene pyramiding). In the current study, the seedling stage submergence responses were assessed based on the survival percentage after submerging 14 day old seedlings for a period of 14 days followed by a de-submerged recovery period of 14 days. The survival percentage ranged between 6 - 97% in the rice panel (Figure 3; Supplementary file S3). Out of the 64 Sri Lankan rice accessions/varieties evaluated for submergence stress responses, 13 rice accessions/varieties showed no significant difference ($p > 0.05$; Figure 3) to FR13A (the submergence tolerance reference), and hence, was grouped as 'tolerant' to submergence stress (survival percentage 77 – 93%). Among the 13 rice accessions/varieties in the 'tolerant' group, eight SLNIVs (At 362, Bg 310, Bw 363, At 303, Bg 359, Bg 369, At 308 and Bg 352) and five SLTAs (*Maa wee*, *Heenkarayal*, *Godaheenati, Batapola el,* and *Dik wee*) were identified.

Among the submergence susceptible group, 13 rice accessions/varieties were reported as having similar responses to *Swarna*, the susceptible reference cultivar ($p > 0.05$; SLNIVs Bg 250, Bw 14-509, Bw 272-6b, Bw 12-574, Bg 94-1, Bw 400, At 307, Bg 300, Bw 351, Ld 368 and Bw 11-3403; and SLTAs *Podihatatha,* and *Sudugoda wee*; Figure 3). Further, 12 rice accessions/varieties were reported as prone to submergence even more than *Swarna* (SLNIVs Bg 251, Bg 302, At 373, Bw 451, Ld 253, Bw 367, Bg 360, Ld 365, Ld 371 and At 311; and SLTAs *Dahanala* and *Kuru wee*; Figure 3). Hence, altogether 25 rice accessions/varieties were reported as 'susceptible' (survival percentage 6 - 57%; Supplementary file S3). The remaining 26 rice accessions/varieties (11 SLNIVs and 15 SLTAs) are grouped as "intermediate" in their submergence response as they were neither as tolerant as FR13A, nor as susceptible as *Swarna,* the tolerant and susceptible reference cultivars, respectively (Figure 3; Supplementary file S3).

Out of the SLTAs *Godaheenati* has been previously reported as tolerant to submergence (Xu & Mackill, 1996; Xu *et al.*, 2006; Bailey-Serres *et al.*, 2010; Panda *et al.*, 2021) and hence is in agreement with the current study. Though Ranawake *et al.* (2014a) reported evaluation of several SLNIVs for submergence tolerance, the survival rates of the said experiment were extremely low compared to the current study; therefore, the two studies could not be compared meaningfully. Moreover, given that submergence stress tolerance is a quantitative trait, it is noteworthy that variations in submergence stress responses can be expected in varieties/accessions across experiments due to the differences in experimental and environmental conditions (Ranawake *et al.* 2014b; Rathnayake *et al.* 2012; Rupasinghe *et al.* 2016).

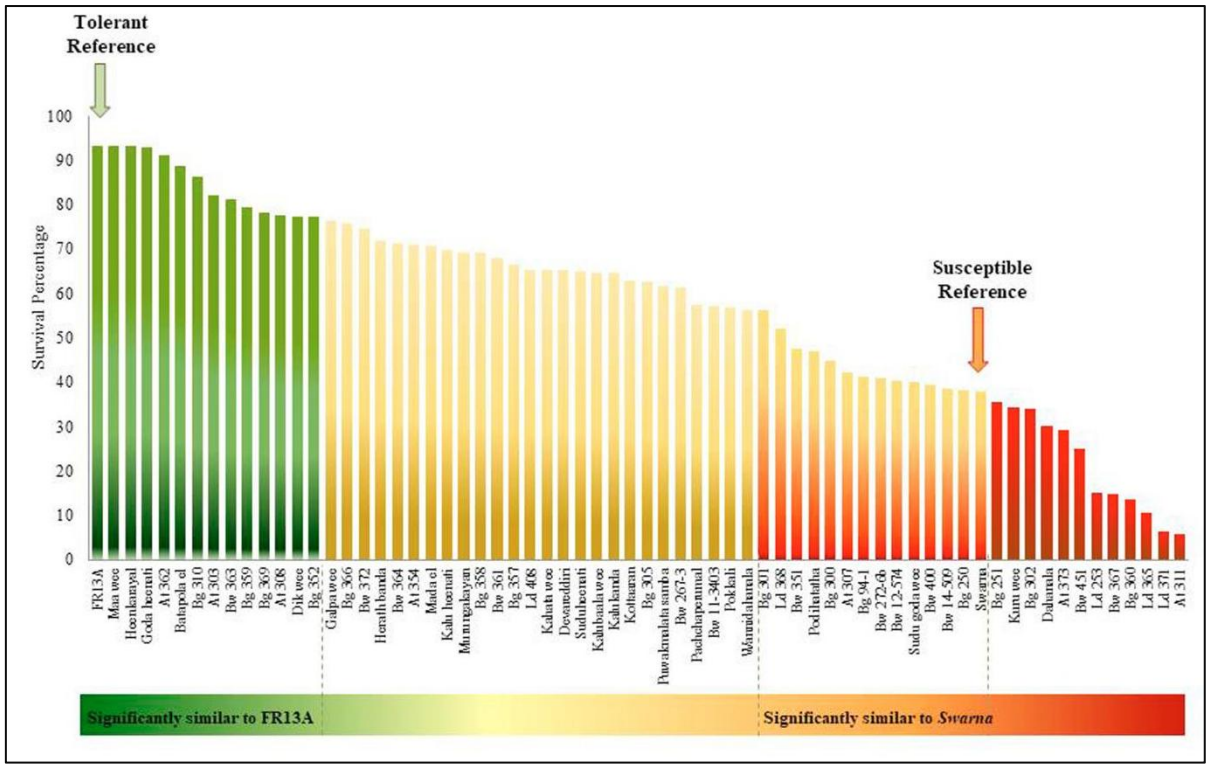

Figure 3: Illustration of submergence stress responses of 64 rice accessions/varieties based on their estimated median scores

Association of *Sub1A* alleles with submergence stress responses

Among the rice accession/varieties confirmed to be carrying *Sub1A* alleles (13 with *Sub1A-1* and 19 with *Sub1A-2*) no significant association ($p < 0.05$) could be found with respect to their submergence stress response in the field trial (Figure 4). Of the 13 rice accessions/varieties reported as tolerant to submergence in the current field study, only *Maa wee*, *Heenkarayal, Godaheenati* and *Dik wee* were confirmed to carry *Sub1A-1.* The tolerant accessions/varieties *Batapola el,* At 362, Bg 359, Bg 369, and Bg 352 carried *Sub1A-2.* Further, the *Sub1A* allele status of the varieties Bg 310, At 303, At 308, and Bw 363 remain unconfirmed (as the possibility that some of them are not carrying the *Sub1A* gene could not be definitively ruled out; Supplementary file S3). These rice accessions/varieties, which do not carry the *Sub1A-1* allele, yet express a tolerant response to submergence, could be carrying other known tolerant alleles such as *Sub1C-1* (which could mask the susceptible response of *Sub1A-2* (Xu *et al.*, 2006) or novel QTL/genes unique to the Sri Lankan rice germplasm. Therefore, it is recommended to study the genetic background of such accessions/varieties to further understand the underlying genetic control of submergence stress responses through QTL mapping/gene discovery.

Of the 25 rice accessions which reported a submergence susceptible response similar to or more severe than *Swarna*, three (Bw 272-6b, *Sudugoda wee* and *Podihatatha*) reported the presence of *Sub1A-1* allele and five (At 307, Bw 400, Bg 94-1, Bg 250 and *Kuru wee*) reported the presence of *Sub1A-2* allele. The allele calls of the remaining 17 rice accessions/varieties (Supplementary file S3) could not be confidently confirmed as the absence of *Sub1A* gene or a PCR failure. The reported susceptibility to submergence stress in the three rice accessions carrying the *Sub1A-1* allele indicates that there could be other genetic factors governing submergence tolerance than the impact of *Sub1A* locus alone (*i.e.*, susceptibility factors). Therefore, further investigations on the genetic mechanism underlying the susceptibility expressed by the three rice accessions Bw 272-6b, *Sudugoda wee* and *Podihatatha* will be beneficial.

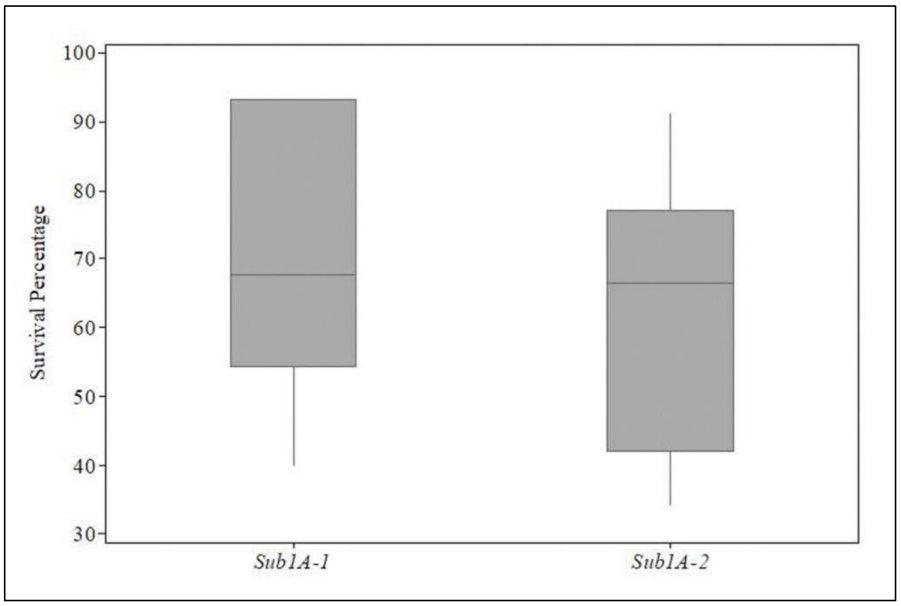

Figure 4: Box and Whiskers plot illustrating the submergence stress response of rice varieties/accessions carrying the *Sub1A-1* and *Sub1A-2* alleles.

CONCLUSION

Allele characterization using the modified gel-based marker *ABUOP0003* revealed the presence of the *Sub1A-1* tolerance allele in the SLNIVs At 354 and Bw 272-6b, and the SLTAs; *Maa wee*, *Heen Karayal, Godaheenati, Dik wee, Mada el, Dewareddiri, Pokkali*, *Pachchaperumal*, *Kahata wee*, *Podihatatha, Sudugoda wee*. Further, nineteen accessions/varieties carried the susceptible *Sub1A-2* allele, and the allele call of the remaining 32 rice accessions/variety could not be confidently verified as absence of *SubA* gene or a PCR failure. The Bayesian genetic structure analysis revealed that the rice panel consisted of two genetically isolated gene pools, in which the occurrence of the *Sub1A* alleles did not show a strong association to the proposed genetic structure grouping. This indicates an allelic divergence of the *Sub1A* gene, which pre-dates germplasm introductions and varietal development attempts in Sri Lanka**.** In a field trial, the SLNIVs; At 362, Bg 310, Bw 363, At 303, Bg 359, Bg 369, At 308 and Bg 352 and SLTAs; *Maa wee*, *Heenkarayal*, *Godaheenati, Batapola el* and *Dik wee* were identified with similar submergence tolerance responses as with the submergence tolerant reference FR13A. Of the remaining rice varieties/accessions, 25 were reported as submergence susceptible, while 26 were reported with intermediate responses to submergence stress. No significant association of the presence of the *Sub1A* allele (*Sub1A-1* and *Sub1A-2*) could be deduced with their respective submergence stress responses. However, given that the SLTAs *Maa wee*, *Heenkarayal*, *Godaheenati* and *Dikwee* carried the *SubA-1* tolerance allele and produced a submergence tolerant stress response, these could be recommended as suitable genetic donors for rice breeding programmes.

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