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RESEARCH ARTICLE

Morpho-physiological screenings and molecular analysis of West Sumatra rice genotypes under submergence stress

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Abstract

This study investigates the submergence tolerance level of 14 rice genotypes by morpho-physiological and molecular analyses of *Sub1* alleles. IR64-*Sub1* was used as a submergence tolerant check. The submergence screenings were conducted by submerging 14-days-old seedlings in water tanks for 14 days while molecular analysis was conducted using 14 *Sub1* linked markers. The results showed that 5 tested genotypes, namely Inpari 48 Blas, Mundam Putiah, Batang Piaman, Banang Pulau and Pulau Sijunjung, recorded high survival rates (SR) of 80% to 100% under submergence stress. PCR-based identification of the *Sub1* alleles confirmed that several tested genotypes carry *Sub1A* and *Sub1C*, but not all were expressed in their phenotypic performance towards submergence. IR64-*Sub1* and Batang Piaman not only showed well-adaptation towards submergence by maintaining lower elongation (<20%) and less chlorophyll content change (TCC) (<30%) but were also equipped with the *Sub1A* allele. These genotypes potentially provide good phenotypic and genotypic performance under submergence stress conditions. Additionally, based on population structure analysis, these genotypes were grouped into 3 clusters, of which 35.71% are pure accessions, while the remaining 64.29% have admixture ancestry between populations 1, 2 and 3. The data in model-based population structure and UPGMA dendrogram supported that rice genotypes in this study have 3 well-differentiated genetic populations and admixtures. Most genotypes have a close genetic relationship with *Nei's* similarity index ranging from 0.571 to 0.893.

Keywords

Morpho-physiological, molecular analysis, rice, submergence, West Sumatra

Introduction

West Sumatra is located at 0°54' N-3°30'S altitude and 98°36'-101°53'E longitude in the middle of the western coast of Sumatra, with an area of 42,130.82 km^2. West Sumatra is one of the centers of rice diversity, which might serve as valuable genetic resources for future rice improvement and provide society's demand for food. Several lowland rice landraces from West Sumatra, such as Anak Daro, Pulau Batu, Cantik Manis, Mundam Putiah, Kuriak and Pulau Sijunjung, are preferred by local consumers due to their good taste and high amylose content (>20%) (1). Therefore, most rice farmers in this region prefer to cultivate these landraces although they are usually low-yielding and long maturity. Socially, local rice varieties are precious. Economically, attempting local rice will increase farmers' income.

Therefore, these local varieties are highly potential for rice improvement, including breeding programs to develop superior rice lines. The high density of rainfall and duration in lowland areas of West Sumatra due to the tropical monsoon climate often leads to flooding disasters, especially in Pasaman regency (2).

Although rice requires much water for growing, exceeding water can negatively affect its growth and lead to yield loss. Besides, submergence also disturbs metabolism processes, such as nutrient uptake, oxidative reactions, respiration, photosynthesis and transpiration. Submergence can reduce seed germination, while extended periods of complete submergence for seven days may cause ultimate death (3, 4). Fortunately, several rice varieties have strategies to cope with this condition. Unlike intolerant varieties, these varieties have genes responsible for the submergence condition's defense. *Sub1* is a major quantity trait locus (QTL) on chromosome 9 near the centromere that is responsible for contributing to rice's submergence tolerance. *Sub1* encodes an ethylene-responsive-factor-type transcription factor (ERF) that promotes abscisic acid (ABA) degradation and increases the production of gibberellic acid (GA). This QTL is associated with carbohydrate consumption, ethanol fermentation, and cell expansion (5). The *Sub1* region contains a cluster of 2 or 3 *Sub1* genes (*Sub1A, Sub1B*, and *Sub1C*) and genotypic variations in submergence tolerance. *Sub1A* cannot be found in all rice with a *Sub1* locus, while *Sub1B* and *Sub1C* can be found in all surveyed rice accessions in this study (6). *Sub1A* maintains the inhibition of GA-mediated growth responses directly through two GA-signaling repressor proteins, SLENDER RICE 1 (SLR-1) and SLENDER RICE-LIKE 1 (SLRL-1), or indirectly through the brassinosteroid pathway. Under submergence, *Sub1A* limits ethylene-activated elongation growth by augmenting GA repressors SLR-1 and SLRL-1 (which limit GA responsiveness) and enhances GA catabolism by differentially regulating the genes associated with brassinosteroid synthesis, inducing a GA catabolic gene. Both processes limit GA-induced growth by conserving carbohydrates for maintaining metabolism and recovery (7).

Even though Indonesian local rice cultivars have been cultivated for a long time, only a few studies have been recorded until now, particularly from West Sumatra. Morphological and molecular characterization of rice accessions with varied genetic backgrounds is essential, especially for their utilization in rice improvement programs (8). The information related to their resistance to biotic and abiotic stress factors is still lacking, as well as their genetic information. Local rice germplasms have been recognized as potentially valuable resources (traits) for rice improvement. Therefore, genetic information and traits such as abiotic and biotic stress resistance are significantly necessary. This study was carried out to determine the submergence tolerance level amongst West Sumatra rice genotypes and to confirm the presence of *Sub1* alleles in the tolerant genotypes using the *Sub1* linked markers. The results of this study can be utilized for future rice improvement programmes.

Materials and Methods

The study was conducted from July 2021 until January 2022 in Pasaman Regency, West Sumatra for the field work, while the laboratory analyses were conducted in the Biotechnology and Plant Physiology Laboratory, Agrotechnology Department, Andalas University, Padang, West Sumatra, Indonesia.

Plant materials

Thirteen (13) rice genotypes, which consist of nine landraces and 4 modern cultivars originated from West Sumatra and a submergence tolerance check genotype, IR64-*Sub1* were used in this study. The details of these rice genotypes are listed in Table 1.

Table 1. The list of rice genotypes used in the study (also represent rice genotypes number in Fig. 3)

No	Genotype	Rice type	Description
1	Pulau Batu	Indica	Local rice, landrace
2	Cantik Manis	Indica	Local rice, landrace
3	Bungo Sungkai	Indica	Local rice, landrace
4	Kuriak	Indica	Local rice, landrace
5	Mundam Putiah	Indica	Local rice, landrace
6	Inpari 48 Blas	Indica	Modern rice
7	IR64-*Sub1*	Indica	Modern rice, submergence tolerant check
8	Inpago 9	Japonica	Modern rice
9	Si Kuniang	Indica	Local rice, landrace
10	Anak Daro	Indica	Local rice, landrace
11	Batang Piaman	Indica	Modern rice
12	Banang Pulau	Indica	Local rice
13	PB-42	Indica	Modern rice
14	Pulau Sijunjung	Indica	Local rice, landrace

Experimental design and submergence treatment

The submergence screening was conducted in a Randomized Complete Block Design (RCBD) with three replications. The comprehensive study to determine the rice submergence tolerance level was conducted following standard methods (9, 10), with some modifications. Ten seedlings of each genotype with uniform growth were sown in each replication. 14-days-old seedlings were fully submerged in water tanks (120 cm in height) for 14 days. Desubmerging was done on the 15th day. After being desubmerged, plants were allowed to recover for an additional 10 days before the final survival rates were recorded. During the submergence treatment, environmental factors such as temperature, turbidity, pH, and dissolved oxygen level were measured.

Morpho-physiological observations

The phenotypic data were measured before and after the submergence treatment (9, 11). Phenotypic data such as plant height (PH) and chlorophyll content (CC) were recorded to obtain elongation percentage (EP) and chlorophyll content changes (TCC). The chlorophyll concentra-

tion of each sample was analyzed using the acetone 80% titration method (12) with spectrophotometry measurement. The fresh shoot samples of the plant were ground to a fine pellet and extracted using 80% acetone. The extract was then used for chlorophyll analysis, followed by the measurement of absorbance at 645, 652 and 663 nm using a spectrophotometer. The survival rate (SR) was evaluated on the 10th day after being de-submerged. The rice tolerance scale was determined by the following IRRI standard (13), which was: scale 1 (survival rate of 100%, tolerant); 3 (survival rate 95-99%, tolerant); 5 (survival rate 75-94%, moderately tolerant); 7 (survival rate 50-74%, moderately susceptible); 9 (survival rate 0-49%, highly susceptible).

Identification of Sub1 in rice genotypes

The leaf samples from 14-days-old seedlings of each rice genotype were collected for DNA extraction. Leaf sampling was conducted from the field around 8 am to avoid wilting. The leaf samples were inserted into the labelled plastic bags for cold storage. Next, genomic DNA was extracted using the GeneaidTM Genomic DNA Mini Kit (Geneaid Biotech Ltd.). The DNA concentration was measured using the BioDropTM spectrophotometer nanodrop and the DNA extracts were stored at -20 ^{0}C. To determine the presence of *Sub1* in the rice genotypes, 14 SSR, InDel and specific markers have been used (Table 2). The markers were developed based on the DNA sequences that had been published earlier (4, 14, 15). The BLAST search was used to find regions of similarity and verify the specificity of the markers for the *Sub1* target region. PCR amplification has been performed by using KOD OneTM PCR Master Mix-blue (Toyobo) following this program: 94 ^{0}C for 5 min (initial denaturation), followed by 35 cycles at 94 ^{0}C for 30 s of denaturation, 35 cycles at 55 ^{0}C for 30 s (annealing), continuing at 72 ^{0}C for 30 s (extension) and final extension at 72 ^{0}C for 7 min. PCR products with selected markers were analyzed by 1.5% agarose gel electrophoresis. After that, the DNA bands were scored to determine the presence of the *Sub1* QTL and to arrange genetic structure and relationships between rice genotypes.

Data analysis

The collected data was analyzed using Minitab v14 software. Analysis of variance (ANOVA) with Tukey's Posthoc test was performed to compare variation between rice genotypes. Pearson's correlation analysis was also performed to determine the correlation between traits. A Fisher Least Significant Difference (LSD) method was used to compare means between factors. The genetic structure was analyzed using the Structure Harvester Program (16), while the genetic relationship and genetic distance were established using NTSYSpc 2.11a software according to Nei's minimum genetic distance matrix (17).

Table 2. The list of molecular markers used for detecting the presence of *Sub1* QTL/genes

No.	Gene	Marker	Marker type	Sequences	Expected product size (bp)	Annealing temperature (℃)
1.	*Sub1C promoter*	ART5	InDel	F:5'CAGGGAAAGAGATGGTGGA3' R:5'TTGGCCCTAGGTTGTTTCAG3'	217	60
2.	*Sub1A (Functional SNP)*	AEX	Mismatch	F:5'AGGCGGAGCTACGAGTACCA3' R:5'GCAGAGCGGCTGCGA3'	231	62
3.	*Sub1A*	SC3/RM8300	SSR	F:5'GCTAGTGCAGGGTTGACACA3' R:5'CTCTGGCCGTTTCATGGTAT3'	211	55
4.	*Sub1A*	Sub1A203	STS	F: CTTCTTGCTCAACGACAACG R: AGGCTCCAGATGTCCATGTC	200	55
5.	*Sub1*	RM 5799	SSR	F: CTTGCACAAGAGGCAACACTCC R: GTTTGGTAGGTCGCATTGTTTGG	146	59
6.	*Sub1*	RM 23843	SSR	F: CCTAGGCCATACATAATCTGACG R: TTAGCGTGGAACTAAACACAGC	585	56
7.	*Sub1*	RM 23915	SSR	F: GAGGATCCTTACCATCAAACTTCG R: CCAAGAACCTGCATTCTTCAAGG	197	56
8.	*Sub1*	RM 23835	SSR	F: TTCCGCTGTTTCTCTTCTTGTGC R: CTGGTTCTGCTGGTTCTGTAGTTGG	193	59
9.	*Sub1*	RM 23865	SSR	F: TCATCCCATTCTCTTCCTCACC R: CATACGGCCATACAAATGAACC	148	56
10.	*Sub1*	RM 23869	SSR	F: GGCATATTCGTGTTGTCCTCACC R: GCCACGCGTACCTGAGATATGG	173	59
11.	*Sub1A promoter*	IYT1		F: TAGGGGCCCATGAGTACTTG R: TCAGACAGCTAGCTCGCAAC	305/554	55
12.	*Sub1A promoter*	IYT3		F: GTTGATAACCGGAGGAGACG R: GTAACCCGACTGGTCTCAGG	305	55
13.	*Sub1C promoter*	ART3	InDel	F: TCTGAACCGGATCATCATTG R: AGTTTGTCTCCATTCGAAGTCA	337	55
14.	*Exon of Sub1C*	Sub1C173	InDel	F: AACGCCAAGACCAACTTCC R: AGGAGGCTGTCCATCAGGT	158-176	55

Results and Discussion

Morpho-physiological analysis

The rice genotypes were grouped into 4 tolerance categories based on phenotypic analysis. The proportions of genotypes that belonged to highly susceptible were 35.71%; meanwhile, moderately susceptible, moderately tolerant and tolerant were 21.43% respectively (Fig. 1). According to the submergence test result, the lowest SR was observed in Anak Daro (20%), which was significantly different from other genotypes, which indicated that this genotype was highly susceptible to submergence (Table 4).

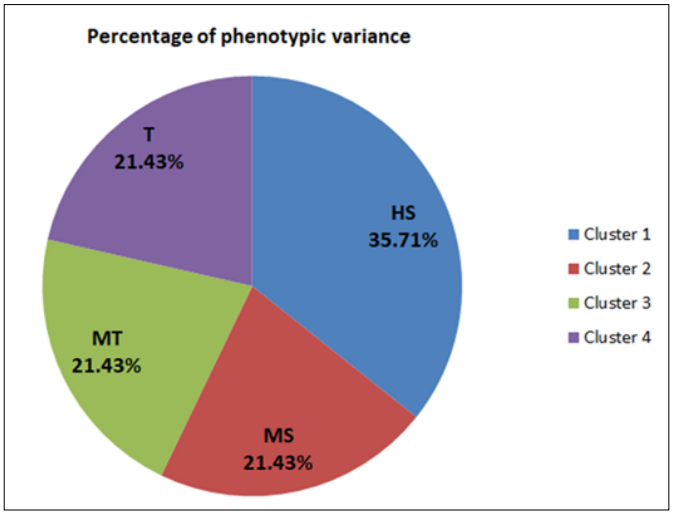

Fig. 1. The clustering composition of 14 rice genotypes based on phenotypic test towards submergence (**HS**: highly susceptible; **MS**: moderately susceptible; **MT**: moderately tolerant; **T**: tolerant).

Tolerant check IR64-*Sub1*, along with Inpari 48 Blas and Mundam Putiah had the highest SR (100%), followed by Banang Pulau, Batang Piaman, and Pulau Sijunjung, which had a SR of 80%. These 3 genotypes belong to the moderately tolerant category based on the IRRI standard-test (1996). Genotypes Kuriak and Si Kuniang belonged to moderately susceptible genotypes and had a SR of 66.67%. Lower SR was observed in Cantik Manis, Inpago 9, Pulau Batu and Bungo Sungkai. The genotypes Inpari 48 Blas and Mundam Putiah are irrigated lowland rice with well adaptation to submergence conditions in this study. Meanwhile, IR64-*Sub1* is known as submergence tolerant and has been confirmed to possess a quiescence mechanism to cope with the submergence (14). Therefore, this check genotype can deal with the water stress condition and preserve energy during submergence conditions.

There are 2 coping mechanisms in plants to defend themselves against submergence. In the escape mechanism, plants are required to elongate the shoot faster to reach the water surface. This mechanism is effective when floodwater rises gradually and persists in the field for a period longer than three weeks and also when the vigour of underwater elongation is sufficient to retain the upper leaves above the water level over periods of up to 37 days (6). Otherwise, some rice species tend to suppress shoot elongation during submergence. The plant can reserve carbohydrates for 10-14 days of submergence in this mechanism and continue its growth processes when the water recedes using energy obtained from the carbohydrate reserves. The *Sub1A* QTL is responsible for this quiescence mechanism. In response to the submergence, *Sub1A* is strongly induced in the tolerant cultivars, whereas intolerant cultivars had weak or no gene induction (18).

In this study, tested rice genotypes underwent morphological changes during submergence, including leaf colour (green turned to yellow), leaf size, stem and root weakness, and sometimes leading to death. Significant differences amongst tested genotypes were observed

Table 4. Morpho-physiological observation results of 14 rice genotypes during submergence screening

Genotype	SR (%)	PHB (cm)	PHA (cm)	EP (%)	TCC (%)	Tolerance scale	Tolerance category
Anak Daro	20.00±0.00g	19.83±2.13bcd	23.73±3.07def	19.51±4.20def	5.09	9	Highly susceptible
Banang Pulau	80.00±20.00abc	19.33±0.253cde	22.47±0.1528ef	16.21±0.791def	27.78	5	Moderately tolerant
Batang Piaman	80.00±20.00abc	23.00±1.253a	26.37±5.19cde	15.5±27.1ef	23.53	5	Moderately tolerant
Bungo Sungkai	46.67±11.55def	15.70±0.557f	23.42±1.665def	49.19±8.76abc	61.95	9	Highly susceptible
Cantik Manis	33.33±11.55fg	19.07±1.76de	28.65±3.96bcd	50.06±12.73abc	59.34	9	Highly susceptible
Inpago 9	40.00±0.00efg	21.91±0.967ab	34.47±1.501a	57.33±4.21ab	62.55	9	Highly susceptible
Inpari 48 Blas	100.00*ab	19.07±1.332de	23.93±0.602def	26.05±12.12cdef	70.33	1	Tolerant
IR64-*Sub1*	100.00±0.00a	16.92±1.467ef	19.31±1.432f	14.41±8.37ef	03.44	1	Tolerant
Kuriak	66.67±11.55bcd	17.37±0.802ef	30.00±3.97abc	73.5±29.7a	18.00	7	Moderately susceptible
Mundam Putiah	100.00±0.00a	17.15±1.169ef	26.61±4.73cde	56.8±36.5ab	61.50	1	Tolerant
PB-42	60.00±20.00cde	20.53±0.642bcd	23.83±3.00def	15.90±12.04ef	78.22	7	Moderately susceptible
Pulau Sijunjung	80.00±28.3abc	19.20±2.51de	20.87±3.53f	8.29±4.69f	28.60	5	Moderately tolerant
Pulau Batu	46.67±11.55def	21.73±1.90abc	29.93±1.371abc	38.09±6.57bcde	66.75	9	Highly susceptible
Si Kuniang	66.67±23.1bcd	22.13±1.553ab	31.98±4.70ab	44.9±21.8abcd	51.59	7	Moderately susceptible
ANOVA	***	***	***	***			

***= significantly different at the level of 1%, Means that do not share a letter are significantly different

for SR, plant height before treatment (PHB), plant height after treatment (PHA), and elongation percentage (EP)

Table 3. The summaries of Analyses of Variance *(ANOVA)* of SR, PHB, PHA and EP

Source	DF	SR	PHB	PHA	EP
Genotype	13	1797.2***	14.498***	57.87***	1279.1***
Error	28	213.2	2.088	10.21	295.1
Total	41				

***= the number significantly different between genotypes at the level of 1%

(Table 3). EP is a valuable morphology parameter used in submergence screening (11, 19). It indicated plant response to environmental changes morphologically. Cell division and elongation are influenced by phytohormone, which is sensitive to the changes. In this study, the EP (Table 4) was different between genotypes. Statistical analysis showed that PHA and PHB are significantly different (p-value ≤ 0.001). Kuriak has the highest EP (73.5%) among all tested genotypes, which was significantly different from others, except Inpago 9, Mundam Putiah, Bungo Sungkai and Cantik Manis. Pulau Sijunjung, IR64-*Sub1*, Batang Piaman, PB-42, Banang Pulau and Anak Daro did not significantly elongate during the submergence treatment (EP < 20%). However, before treatment, the rice genotypes were diverse in agronomical performances, and their PH was significantly different. The changes in PH provide information that the rice genotypes tried to adjust their morphological features in problematic conditions. Meanwhile, 4 genotypes: Pulau Sijunjung, Batang Piaman, PB42 and Banang Pulau, tend to suppress their elongation (did not elongate more than 20%) during submergence and instead elongate faster to reach the water surface. This observation indicated that these genotypes might cope with submergence by the exact mechanism like the tolerant genotype IR64-*Sub1*, a quiescence mechanism. Therefore, the plant can efficiently save energy in water stress conditions by suppressing the organ's elongation. Different mechanisms occur in Mundam Putiah, which this genotype elongates quickly when submerged, reaching up to 56.8%. In fact, Mundam Putiah was also recognized to have a high SR during submergence but did not show a mechanism such as IR64-*Sub1*. Therefore, this genotype's coping mechanism was assumed as an escape strategy.

Chlorophyll content (CC) measurement is necessary to investigate plants' physiological responses to submergence. In this study, significant differences amongst 14 rice genotypes were observed for TCC (p-value ≤ 0.05) (Table 4). PB42 had the highest TCC (78.22%), followed by Inpari 48 Blas, Pulau Batu, and Inpago 9. Meanwhile, IR64-*Sub1* showed less TCC (3.44%), followed by Anak Daro (5.09%), Kuriak (18%) and Batang Piaman (23.53%). This result indicated that submergence treatment significantly influenced the CC of the rice plant. As a crucial photosynthetic pigment, the loss of chlorophyll will affect the plant's photosynthesis process. The light intensity will be reduced when the plant is submerged and it might not reach the underwater leaves' surface. Consequently, the leaves experienced chlorosis and leaf senescence (3). In addition, the plant uses chlorophyll to trap solar energy before water hydrolysis can proceed. Therefore, the significant reduction in CC can ruin photosynthesis and respiration processes and eventually limit the plant growth.

Based on our results, rice genotypes showed different responses to the submergence phenotypically. Based on the phenotypic tests, most rice genotypes tested in this study (35.71%) were highly susceptible, 21.43% were moderately susceptible, 21.43% were moderately tolerant and 21.43% were tolerant (Fig. 1). The data was obtained from the SR observation, as well as the EP and CC observations. Based on those data, most of the local rice genotypes in this study belong to a highly susceptible category.

In water stress conditions, intolerant rice plants usually show several physiological damages, including oxygen depletion, increased elongation process, declined stored energy and cannot return when the water recedes (20). The majority of the local rice genotypes tested (60%) had higher SR (>80%), while five genotypes (Pulau Sijunjung, Batang Piaman, PB42, Banang Pulau and Anak Daro) had low EP (20%) (Table 4). Generally, submergence causes damage to the leaf organ, root and shoot. Since the amount of oxygen that reaches the root via aerenchyma is reduced, the nutrient uptake and development will also be limited. The decline of photosynthate and ATP production limits the energy for shoot and root growth. Despite this constraint, intolerant cultivars typically accelerate the stem and leaf elongation rate relative to non-submerged plants, which resembles partially submerged deepwater rice with pronounced and effective internodal elongation. If the flood is deep, elongating leaves may fail to reach the air-water interface because of exhausted energy reserves (21). However, the escape strategy performed by the rice plant consequently caused excessive carbohydrate consumption and led to an energy crisis. Therefore, after the water recedes, the plant will fail to continue its growth.

According to the data in this study, the genotypes such as Pulau Sijunjung, Banang Pulau and Batang Piaman have been assumed to adopt a quiescence mechanism (as IR64-*Sub1*) to cope with the submergence by having high survival rate, suppressing elongation and maintaining chlorophyll degradation (Table 4). Meanwhile, the susceptible and moderately susceptible genotypes could not avoid submergence impacts by showing lower values of SR, elongated fast, and CC reduction and were eventually damaged by the submergence.

Based on this study, the plants that suppressed elongation and maintained CC during submergence potentially tend to survive. When the plants were submerged, they displayed constrained leaf and internode elongation, chlorophyll degradation and carbohydrate consumption (5). With the decline of light intensity in an underwater environment, plants require more light trappers (photosynthetic pigments) for the photosynthesis process. Chlorophyll a has a crucial role as an electron donor in the electron transport chain, while chlorophyll b has a role in giving organisms the ability to absorb a higher frequency of blue light for use in photosynthesis.

The underwater organ's metabolism is different from the non-submerged organ. Submerged leaves tend to have a low photosynthetic capacity on an area basis, matching the low rates of supply of CO_2 and light (22). The availability of light and gas exchange affected the regulation of metabolism underwater. Reduction in light intensity and gas diffusion are major challenges for the plant in submergence conditions (23). The complexity of metabolism affects plant growth and survival during submergence. The plant genotypes treated by submergence in this study display different phenotypic performances, which are assumed to be the result of metabolic changes as the response to the submergence stress. However, additional experiments are required to investigate physiological response and its relationship to the presence of the genes and their expression.

Identification of Sub1 alleles

Of the 14 *Sub1* linked markers used in this study, ten markers were polymorphic. Meanwhile, other DNA markers, such as ART-3, ART-5 and Sub1C173, were linked to Sub1C. AEX is a flanking marker designed specifically for tolerant alleles, while IYT-1 and -3 are CAPS markers designed as promoter regions of *Sub1A*. Sub1C173 was designed to differentiate the allele of *Sub1C*, while ART-3 and -5 were promoters for that region. SC3 was considered a downstream marker for the *Sub1A* region and was developed to differentiate tolerant from intolerant genotypes (14). Moreover, several highly polymorphic markers are tightly linked and located upstream of the *Sub1* locus, e.g., RM23835 (5.5 Mb), RM23865 (6.2 Mb), RM23869 (6.3 Mb), RM5779 (3.7 Mb), RM23843 (5.6 Mb) and RM23915 (7.2 Mb) were used in this study. The primers were used to find a region of similarity and verify the marker's specificity for the *Sub1* target region (15). However, five of them, RM23915, RM23843, RM23865, RM23869, and RM5799, were polymorphic in this study. The IR64-*Sub1* mega-variety has been proven to have *Sub1A* and *Sub1C* genes. By using polymorphic markers linked to *Sub1A* and *Sub1C* genes (AEX, SC3, Sub1A203, ART-5, and Sub1C173), tolerant varieties have been recognized to have *Sub1A* and *Sub1C* genes in this study (Table 4, Table 6), for example, IR64-*Sub1* and Batang Piaman. However, genotypes equipped only with *Sub1C* are usually intolerant to submergence (such as Anak Daro, Cantik Manis and Pulau Batu).

IR64-*Sub1* has a high tolerance level to submergence, with survival rates ranging from 75-87% (14). The marker used in this study, such as SC3, is the closest SSR marker downstream of *Sub1A*. Most markers were used to differentiate the tolerant and intolerant rice genotypes. Furthermore, *Sub1A* has been confirmed as a major determinant of submergence tolerance, whereas the alleles of *Sub1C* genes did not significantly affect the tolerance level. Additionally, *Sub1* QTLs were identified on chromosome 9 and they contribute up to 70% of phenotypic variation in tolerance (24). Several independent studies confirmed the major chromosome 9 QTL and also identified other minor QTLs that accounted for less than 30% of the phenotypic variation in submergence tolerance (25, 26). Therefore, as shown in this study result, the genotypes with the *Sub1A* alleles (using markers AEX and Sub1A203) tend to be tolerant towards submergence. Otherwise, the susceptible genotypes such as Pulau Batu, though they carry the *Sub1C* allele, are still considered submergence susceptible. This demonstrates that the plant cannot cope with the submergence condition without *Sub1A*.

Interestingly, a rice genotype (Inpago 9) was considered to have *Sub1A* alleles (identified using markers SC3 and Sub1A203) and was susceptible to submergence. This fact showed that even with the *Sub1A* allele, not all genotypes could survive in the submergence condition. A recent study, which has developed gene-based and intragenic DNA markers based on DNA sequences, suggested that *Sub1A* is identified in two allele forms in submergence tolerant and intolerant *indica* and *aus* accessions, based on nucleotide variations in the protein-coding region. *Sub1A-1* is found only in tolerant lines such as FR13A, whereas the *Sub1A-2* allele is present in intolerant *indica* accessions. *Sub1A-1* and *Sub1A-2* encode identical proteins, except for $Serine_{186}$ in the tolerant allele and $Proline_{186}$ in the intolerant allele (4).

In this study, the genotypes with the *Sub1A* allele tend to have a high submergence tolerance level, for

Table 6. The screening results of 14 rice genotypes using 14 DNA markers linked to *Sub1* on chromosome 9

Gene	Marker	Target gene existence in each rice genotype													
		AD	BP	BtP	BS	CM	Ip9	IB	IR	K	MP	PB42	PS	PB	SK
Sub1C promoter	ART5	-	-	+	+	+	-	-	+	+	-	-	-	+	+
Sub1A	AEX	-	-	+	-	-	-	-	+	-	-	-	-	-	-
Sub1A promoter	SC3/RM8300	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Sub1A	Sub1A203	-	-	+	-	-	+	-	+	-	+	-	-	-	-
Sub1	RM5799	+	-	-	-	-	-	-	+	-	-	-	-	-	-
Sub1	RM23843	+	-	-	-	-	-	-	+	-	-	-	-	-	-
Sub1	RM23915	+	-	+	-	-	-	+	+	-	-	-	-	-	+
Sub1	RM23835	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sub1	RM23865	-	-	-	-	-	-	-	-	+	+	-	-	+	-
Sub1	RM23869	-	-	-	+	+	-	+	-	+	+	-	-	+	-
Sub1A promoter	IYT1	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Sub1A promoter	IYT3	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sub1C promoter	ART3	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sub1C promoter	Sub1C173	+	-	-	-	+	-	-	+	+	+	-	-	-	+

example, Batang Piaman and Mundam Putiah. This result showed that although *Sub1A* is responsible for submergence tolerance, several factors are also involved, such as the expression level and dosage of *Sub1A* and other genes that control it. The expression of *Sub1A-1* is stronger than *Sub1A-2* in determining the submergence tolerance level, and the expression of the *Sub1A* allele in heterozygotes was less than its expression in homozygotes (14).

Genotypes without *Sub1A-1* quickly consumed leaf starch and soluble sugars to maintain elongation growth during submergence. *Sub1A-1* promoter activity in the internodes, collar region, and leaf base is constant in suppressing the division and elongation of cells (4). In this study, the genotypes with the *Sub1A* allele consistently suppress their elongation and less chlorophyll degradation during submergence, including IR64-*Sub1* and Batang Piaman. In addition, they also have high SR (80%) and are considered moderately tolerant to submergence. These 2 genotypes have the potential to be used in breeding programme to develop adaptive cultivars in the future.

In submergence conditions, the gaseous plant hormone ethylene is entrapped in the plant tissues to regulate morphological and anatomical flood-adaptive responses. Ethylene plays a vital role in hypoxia anticipation and metabolic acclimation during submergence. Several studies suggest that ethylene has a fundamental role in inducing autophagy and stimulating reactive oxygen species (ROS) amelioration, which contributes to survival during submergence, hypoxia and re-oxygenation stress (27). However, there are differences in ethylene regulation in escape and quiescence mechanisms. In the escape mechanism, ethylene activates the ERFVII transcription factors SNORKEL 1 and 2 in deepwater rice, increasing internode elongation to escape hypoxia by restoring above-water gas exchange (28). Meanwhile, in the quiescence mechanism, for example, in lowland rice, ethylene also actively suppresses shoot elongation through induction of the ERFVII *Sub1A* (4). Under hypoxia conditions, the elongation organ of totally submerged deepwater rice showed vigorous induction fermentation and glycolysis to fuel an escape response (29). Otherwise, ethylene-regulated *Sub1A* limits starch breakdown and carbohydrate metabolism during submergence and post-submergence, enhancing the catabolism of several amino acids such as alanine (30).

The submergence tolerance conferred by *Sub1A* in rice genotypes is supposed to be correlated to the better maintenance of total soluble carbohydrates, limited elongation growth, reduced aldehyde contents, less chlorophyll degradation and less oxidative damage upon re-oxygenation (5, 19, 31). The morpho-physiological and molecular aspects being assumed are conformable to each other. The results of this study supported the facts, although, in phenotypic observation, the correlations were not significant (Table 5). There were several factors that were involved, including the limitation of genetic determinants of the observed variation in metabolic and developmental responses which is still unclear. *Sub1* QTL influenced approximately 35-69% of phenotypic variance in submergence tolerance in diverse background (24, 25).

Genetic structure of rice genotypes

Another important feature to understand about diversity between rice genotypes is genetic structure analysis. Since most of the rice genotypes tested in this study come from different region in West Sumatra, genetic diversity is fundamental because the complexity of genetic variation is influenced by environmental adaptability, yield stability and disease susceptibility of crop species (32). The traditionally cultivated landraces, such as some of the rice cultivars in this study, have been considered essential natural resources to fulfil food demand in the recent climate change situation. Genetic variability also provides significant information for breeding programme. Crosses between populations with different genetic relationship are expected to produce a wide range of variation in yield and other traits (33, 34). Evanno's correction (35), showing the only peak of ΔK for K=3, suggests the presence of three

Table 5. Correlation between studied traits

	PHB	PHA	EP	SR	CCB	CCA
PHA	0.428***					
	0.005					
EP	-0.250	0.759***				
	0.110	0.000				
SR	-0.157	-0.314	-0.190^{ns}			
	0.341	0.052	0.245			
CCB	-0.050	0.341	0.388	-0.290^{ns}		
	0.864	0.232	0.170	0.337		
CCA	0.313	-0.530	-0.744***	0.501	-0.368^{ns}	
	0.275	0.051	0.002	0.081	0.196	
TCC	0.139	0.471	0.393	-0.198^{ns}	0.566**	-0.358^{ns}
	0.636	0.089	0.165	0.517	0.035	0.209

main populations in rice (Fig. 2A, 2B, 2C, 2D). The classification of the genotypes into populations can be seen in the STRUCTURE plot (Fig. 3), where 3 types of allelic combinations in genotypes tested (red-, green-, blue-coloured segments) are produced. Population 1 (P1, red-coloured), 2 (P2, green-coloured) and 3 (P3, blue-coloured) consisted of five (35.71%) pure accessions. The remaining nine genotypes (64.29%) were categorized as having admixed ancestry. There were 3 genotype admixtures between P1 and P2, 2 genotype admixtures between P1 and P3 and 4 genotype admixtures between P1, P2 and P3.

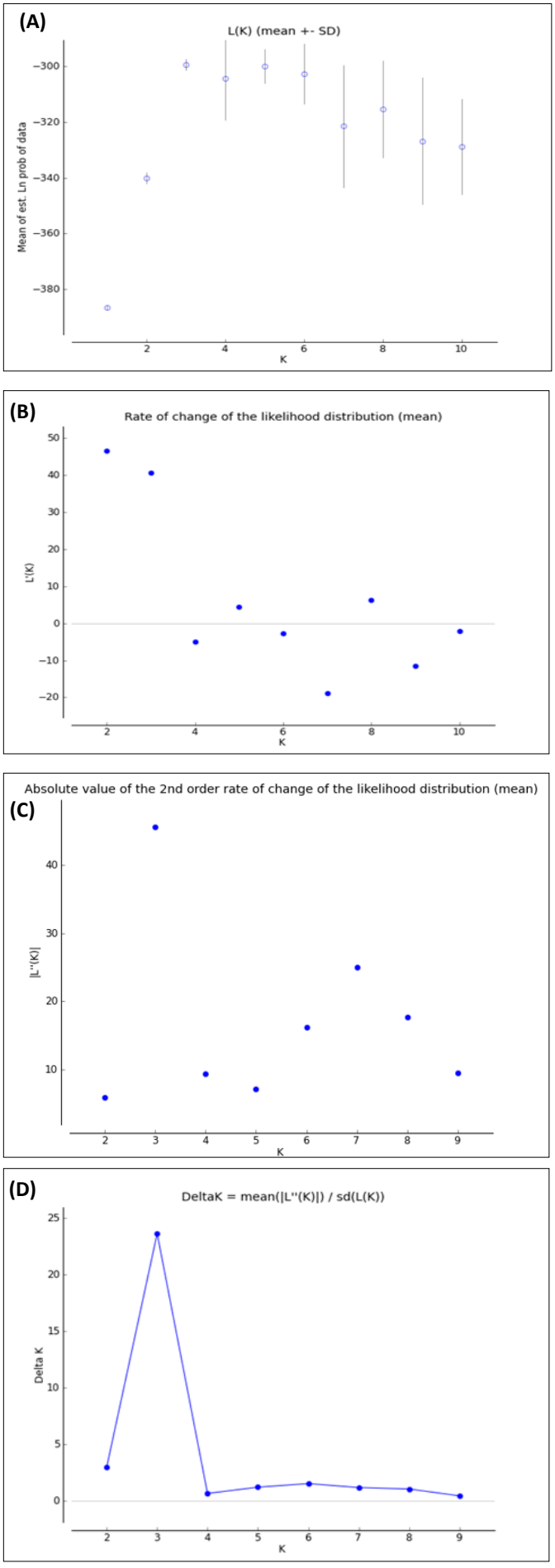

Fig. 2. Delta K-plot of Evanno test.

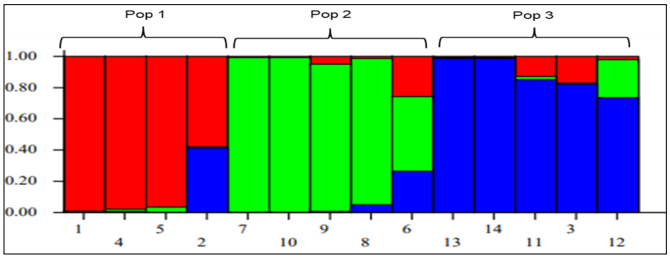

Fig. 3. STRUCTURE plot of 14 rice genotypes showing allelic combinations and their frequencies.

The hierarchal clustering of the UPGMA dendrogram (Fig. 4) is consistent with the flat population structure model. The dendogram indicated that 14 genotypes were clustered into 3 distinctly separated populations. Cluster 1 (the largest) consisted of 2 subclusters, 1a and 1b. Subcluster 1a contained Pulau Batu, Kuriak, Cantik Manis, Bungo Sungkai and Mundam Putiah, while subcluster 1b contained Inpari 48 Blas, Batang Piaman and IR64-*Sub1*. On the other hand, Cluster 2 is composed of four accessions, which are Inpago 9, Banang Pulau, Si Kuniang and Anak Daro. The smallest population consisted of two accessions, PB-42 and Pulau Sijunjung, which were grouped into Cluster 3. Based on this analysis result, the genotypes with a common allele and resistant to waterlogging are almost scattered in the same cluster (for example, subcluster 1b). The tolerant and moderately tolerant genotypes IR64-*Sub1*, Inpari 48 Blas, Batang Piaman and Mundam Putiah are located in the same group. Even though the other susceptible genotypes were also located in the same cluster, Cluster 2 (C2) consisted of 2 highly susceptible, 1 moderately susceptible and one moderately tolerant genotype. In addition, a moderately susceptible and a moderately tolerant genotype (PB-42 and Pulau Sijunjung) are in the same Cluster (C3). From these results, this cluster analysis was not completely able to separate genotypes based on their allelic characteristics.

A dendogram was generated to analyse the relationships between the fourteen genotypes investigated (Fig. 5). *Nei's* similarity index ranged from 0.571 to 0.893. The closest genetic relationship between genotypes can be seen in Cantik Manis and Bungo Sungkai. Furthermore, genotypes IR64-*Sub1* and Inpago 9 have the farthest genetic relationship according to this similarity index. The high similarity index indicates that all the tested genotypes in this study are mostly similar and were close genetically. Although some samples in this study come from common rice varieties released by the government, they mostly have a close genetic relationship with the local rice genotypes (assumed due to the small population size). In summary, popula-

tion structure and UPGMA dendrogram supported the conclusion that the rice genotypes in this study have three distinct genetic populations and admixtures.

ters. The data in model-based population structure and UPGMA dendrogram supported that rice genotypes in this study have three well-differentiated genetic populations and admixtures.

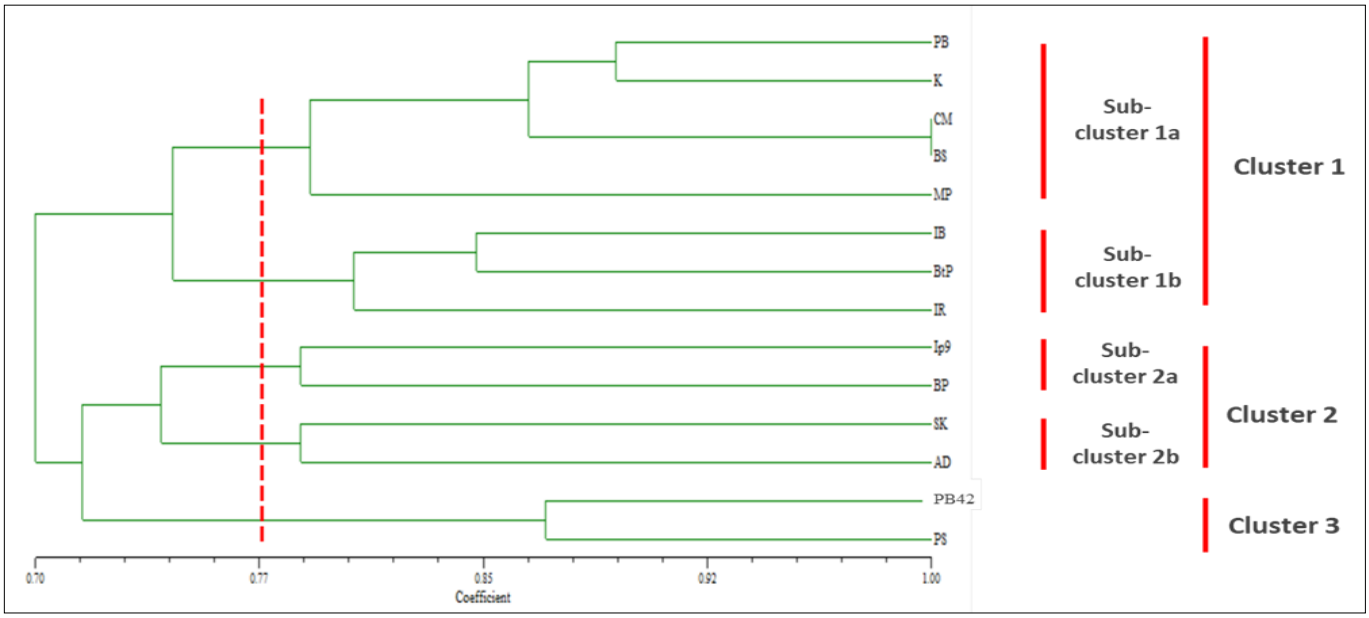

Fig. 4. UPGMA dendogram shows genetic relationship between all rice genotypes (according to 39 loci).

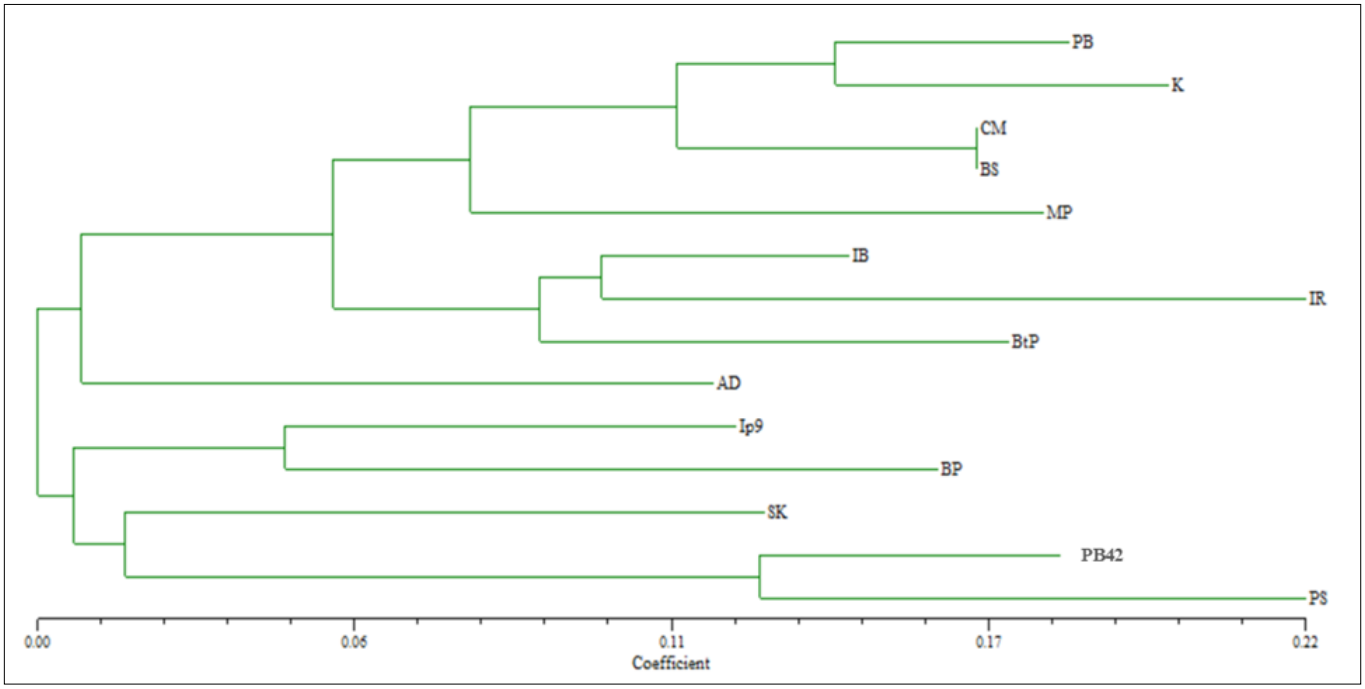

Fig. 5. Genetic distance between 14 rice genotypes.

Conclusion

Based on phenotypic analysis, most of the local rice genotypes cultivated by farmers in West Sumatra are highly susceptible to submergence. The PCR-based identification of the *Sub1* alleles indicates that several genotypes have *Sub1A* and *Sub1C*, but not all were expressed in their phenotypic performance towards submergence. Batang Piaman was found to be the promising rice genotype that showed a high survival rate and was well-adapted towards submergence, especially in the maintenance of shoot elongation and chlorophyll degradation, and it is also equipped with *Sub1A* alleles. Structure analysis indicates that all tested genotypes can be grouped into three clus-

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Authors contributions

SDP carried out the morpho-physiological screenings and molecular genetic studies, statistical analysis and drafted

the manuscript. NAAS participated in the design of the study, performed the statistical analysis, and drafted the manuscript. NLS and JJ conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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