Polymorphism parental survey in three Indonesia improved varieties to support development of new submergence tolerant varieties

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Abstract. Submergence by flooding caused damage in rice growing areas and huge economic loss, and developing tolerant varieties is considered as the best approach to overcome the problem. Markers Assisted Backcrossing (MABC) approach is widely to develop Sub-1 tolerant varieties. The availability of polymorphic markers is among the most crucial requirement to implement the MABC method. This research was subjected to assess DNA polymorphism between IR64Sub1 and three Indonesian popular varieties. A total of 136 microsatellites/simple sequence repeat markers were used to genotype three Indonesian popular varieties; Cisantana, Angke and Mekongga and IR64-Sub1. A total of 39 markers covering 11 chromosomes were found polymorphic between IR64 Sub-1 and the three varieties, however no polymorphic markers found in chromosome 12. The lack of polymorphic markers were also found in chromosome 10 and 11 between IR64 Sub 1 and Angke. With the completion of the missing markers, these 39 polymorphic SSR markers can be utilized to support the MABC program for the development of new Sub-1 tolerant variety with multiple tolerances.

1. Introduction
Flooding is one of the most crucial environmental stresses for crop in many regions. In Indonesia, flood prone areas are existed in all 33 provinces [1] The annual average of the flood areas in Indonesia were about 268,800 ha, including completely damaged areas of about 58,000 ha. Both Flash flood and stagnat flood are not only affected rice production in submergence area, but also in rice irrigated area [2]). Based on the physical exposure, vulnerability, and risk, the disaster risk index of the flood disaster in majority of the provinces in Indonesia are categorized as high [1] and grow in permanently waterlogged soils, but when completely submerged, mostly die within a week [3]. Most rice area in Indonesia are irrigated ecosystem, which covered 59.37% of total rice area, whereas the rainfed and submergence area covered 26.49% and 8.34% of total rice area, respectively [4]. This flood caused damage many hectares of paddy field. From 2000-2008, around 6352.540 - 997.332 ton of dried milled grain rice yield loss because of flood [2].

Developing submergence tolerant rice varieties has a great potential to overcome problems of rice cropping in flood-prone areas. Submergence tolerance rice was successfully developed when the Sub-1 gene from Indian landrace FR13A successfully mapped and introgressed into farmer preferred varieties [5]; [3]. Breeding of submergence tolerant rice was performed by marker assisted
backcrossing (MABC) [6]; [7]. Through this scheme, the Sub-1 locus has been successfully introgressed into modern high-yielding varieties, such as Swarna, Samba Mahsuri, IR64, Thadokkam 1(TDK1), CR1009, and BR11 [7]. Since then, the introgression of this locus region through MABC is widely applied to diverse genetic ackgrounds. New submergence tolerant varieties has been developed in Vietnam [8], Indonesia [9]; [10], Bangladesh [11], India [12], Malaysia [13], and other countries.

MABC is one of the most promising approaches is the use of molecular markers to contribute to develop resistant varieties by incorporating a gene of interest into an elite variety which is already well adapted by the farmers [14]. The strategy of MABC is utilizing molecular markers to assists the transfer of particular allele from a donor line to a recipient line and then selecting against donor introgressions across the rest of the genome using molecular markers [6]. While conventional breeding generally takes at least 10 years to release new variety [15], MABC offer a quickens the advancement of tolerant/resistant cultivars in the lowest number of generations by accelerating the recovery of recipient parent genome [16]; [17]. This approach fastened the recovery of the recurrent parent genotype using only two or three backcrosses [14]. Even, when applying to the closely related parents, new varieties can be achieved through only one backcross [9].

Molecular markers that were tightly linked, flanking, and unlinked to target trait are used to apply foreground, recombinant, and background selection, respectively, in backcrosses between donor and the recurrent parent [6]. Microsatellite markers that were polymorphic between the two parents were used to enhance the combination of the recurrent parent genome with the SUB1 region located on chromosome 9 [6]; [3]. SSR is considered as a reliable, powerful, and transferable between population [15]. This marker is also relatively cheaper and requires a comparatively simple technique with a higher polymorphism rate [17]; [18]. These respective characters determining its suitability for MAS, which among others are reliability, repeatability and reproducible, cost effective, and polymorphic [15]. With these respective advantages, this typical marker is widely used in Linkage and QTL mapping and Marker-assisted [17]; [15].

Inpari 30, the submergence version of Ciherang, has widely adopted by farmers since it was released in 2012. This variety was developed through MABC with IR64-Sub 1 as donor parent [9]; [10], this variety is moderately susceptible to BPH and BLB [19]. Despite of that susceptibility, the strategy can be adopted for the development of new varieties with multiple tolerances. MABC can be planned to introgress sub 1 locus into targeted potential recurrent parent. Cisantana, Angke and Mekongga are among the potential varieties that can be incorporated in this breeding program. These varieties could be converted to Submergence version through MABC.

The first step transferring of sub 1 gene into the certain genotype is study of the polymorphism of the candidate of parental and the donor parent. The objective of this research is to assess the DNA polymorphism between IR64-Sub 1 and tree accessions of Indonesian popular rice varieties by using SSR markers. The information of polymorphic markers would be useful for determining markers suitable for background screening as well as determining genetic relatedness among the varieties. Moreover, this will beneficial to support development of new Sub-1 resistant variety.

2. Material and Methods
The study was performed in 2011 at the Genetic Laboratory of Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD). Four rice varieties consisted of three Indonesia commercial varieties i.e. Cisantana, Mekongga, and Angke and IR64-Sub1 were screened for molecular polymorphism by using SSR markers. IR64-Sub1 seeds were introduced from International Rice Research Institute (IRRI), whereas the three Indonesian commercial varieties were collected from Indonesian Center for Rice Research (ICRR). A total of 136 SSR markers covering all the 12 rice chromosomes were used for polymorphism selection.

DNA was extracted from 3 week old plants according to the protocol of micro-scale rice DNA isolation by Zheng et al. [20]. PCR was performed in 20 ul reactions containing 20 – 50 ng DNA template, 2 ul of 10x TB buffer (containing 200 mM Tris-HCl pH 8, 500 mM KCl, 15 mM MgCl2), 2 ul of 1 mM dNTP, 1ul each of 5 uM forward and reverse primers and 0.3 ul of Taq DNA polymerase.
The amplification was performed on MJ research PCR machine and with a PCR profile performed as below: Initial denaturation for 5 min at 94°C, followed by 35 cycle of 40 s denaturation at 94°C, 45 s annealing at 53°C, and 1 min extension at 72°C with final extension for 5 min at 72°C at the end of cycle.

The PCR product was mixed with 6x bromophenol blue and were analyzed by electrophoresis on 8% polyacrylamide gel using mini vertical polyacrylamide gels of CBS Scientific Co Inc, CA, USA. The gels were stained in 1% Ethidium bromide staining solution for 10 – 20 min and were documented on Chemidoc gel viewer.

The gel view was scored and identified by comparing the band size of DNA fragment of the three varieties and IR64Sub-1. DNA fragments with different size are identified as polymorphic, whereas the same size identified as monomorphic. The genotype data were then arranged and analyzed using NTSYS program to assess the similarity of the accessions.

3. Result and Discussion
A total of 160 markers utilized and 39 markers were found polymorphic between IR64 Sub-1 and three Indonesia popular rice varieties. Those polymorphic markers covering 11 chromosome, except chromosome 12. Chromosomes with highest number of polymorphic markers are chromosome 2, 1, 5, and 7 with the number of 16, 13, 9, 1nd 9 polymorphic markers found, respectively. In these 11 chromosomes, there are at least one marker can distinguished alleles between IR64 sub1 and other varieties. However, there were some gaps found in some particular chromosome (Table. 1).

Different numbers of polymorphic markers were found between IR64 Sub-1 and the three varieties. Highest number was found between IR64 Sub-1 and Cisantana and the lowest number found between IR64 Sub-1 and Angke, with the total of 36 and 22 polymorphic markers, respectively. Whereas, there are 24 polymorphic markers found between IR64 Sub-1 and Mekongga. Aside of the unavailability of polymorphic markers on chromosomes 12, we also lacked polymorphic marker on chromosome 10 and 11 to distinguish IR64 sub 1 with Angke. In chromosome 4, we also lacked of codominant-polymorphic markers to distinguish IR64 Sub-1 and Mekongga. Marker RM 317 produce no band on IR64Sub1 and suppose work as dominant markers. However, this assumption still needs further reconfirmation. Amplification with GAPDH will help to confirm this situation.
Table 1. Polimorphic SSR markers between IR64 sub1 and Indonesian popular varieties

| Varieties          | Chromosome 1 | Chromosome 2 | Chromosome 3 | Chromosome 4 | Chromosome 5 | Chromosome 6 |
|--------------------|--------------|--------------|--------------|--------------|--------------|--------------|
| IR64 sub1-Cisantana| RM 14        | RM 207       | RM 135       | RM 401       | RM 169       | RM 111       |
|                    | RM 212       | RM 279       | RM 156       | RM 317       | RM 249       | RM 162       |
|                    | RM 226       | RM 290       | RM 16        | RM 413A      | RM 225       |              |
|                    | RM 312       | RM 423       |              |              | RM 507       |              |
|                    | RM 493       |              |              |              |              | RM 3         |

| Varieties          | Chromosome 1 | Chromosome 2 | Chromosome 3 | Chromosome 4 | Chromosome 5 | Chromosome 6 |
|--------------------|--------------|--------------|--------------|--------------|--------------|--------------|
| IR64 sub1-Angke    | RM 14        | RM 145       | RM 135       | RM 401       | RM 413A      | RM 162       |
|                    | RM 226       | RM 207       | RM 156       | RM 317       | RM 413B      | RM 507       |
|                    | RM 493       | RM 279       | RM 423       | RM 249       |              |              |

| Varieties          | Chromosome 1 | Chromosome 2 | Chromosome 3 | Chromosome 4 | Chromosome 5 | Chromosome 6 |
|--------------------|--------------|--------------|--------------|--------------|--------------|--------------|
| IR64 sub1-Mekongga | RM 495       | RM 145       | RM 135       | RM 317       | RM 169       | RM 162       |
|                    | RM 14        | RM 207       | RM 156       | RM 231       |              |              |
|                    | RM 212       | RM 279       | RM 290       |              |              |              |
|                    | RM 226       | RM 327       | RM 423       | RM 249       |              |              |
|                    | RM 499       | RM 526       |              |              |              |              |

| Varieties          | Chromosome 7 | Chromosome 8 | Chromosome 9 | Chromosome 10 | Chromosome 11 | Chromosome 12 |
|--------------------|--------------|--------------|--------------|---------------|--------------|--------------|
| IR64 sub1-Cisantana| RM 11        | RM 223       | RM 205       | GT 25         | RM 479       |              |
|                    | RM 214       | RM 264       | RM 316       | RM 474        |              |              |
|                    | RM 20589     | RM 281       | RM 350       |               |              |              |
| IR64 sub1-Angke    | RM 11        | RM 281       | RM 316       |               |              |              |
|                    | RM 214       | RM 25        |              |               |              |              |
|                    | RM 20589     |              |              |               |              |              |
|                    | RM 248       |              |              |               |              |              |
| IR64 sub1-Mekongga | RM 11        | RM 223       | RM 316       | GT 25         | RM 144       |              |
|                    | RM 214       | RM 25        |              | RM 330A       | RM 474       |              |
|                    | RM 20589     | RM 281       |              | RM 474        |              |              |
|                    | RM 248       | RM 350       |              |               |              |              |
Figure 1. Gel view revealed polymorphic markers (A,B,C) and monomorphic markers (D) between IR64Sub1 and Indonesian commercial varieties

Note: 1=IR 64 Sub1, 2=Cisantana, 3=Angke and 4=Mekongga

Marker screening is very essential before starting MABC [16]. The unavailability of polymorphic markers in the chromosome 10, 11 and 12 prevent the implementation of MABC. Aside of the availability of closely linked marker and flanking markers for the target locus, the size of the population, and number of backcross, the effectiveness of MAB is also determined by the number of markers for background selection [16]. The progress of the recovery of the recipient parent genome depends on the number of markers used in this step [21]. Therefore, polymorphic markers covering these three respective locus have to be provided prior to the breeding. Once those are identified, the transfer of sub1 from the IR64 Sub-1 into those three varieties can be initiated. These markers will give view of how much the recovery of the recurrent parent genome in particular BC progeny and to select for the best backcross progeny [16].
The phenogram showed high relationship between the four rice varieties that were analyzed. The three Indonesian commercial varieties were separated from IR64-Sub1 at the similarity level of 0.78%. Basically these three popular varieties were also closely linked to IR64, since IR64 is one of the parents of these varieties. Cisantana was derived from IR 64/IR54742-1-19-11-8, Mekongga was derived from A2790/2*IR64, whereas Angke was derived from IR646/IRBB5. These varieties were released on 2000, 2001, and 2004 for Cisantana, Angke and Mekongga rice varieties, respectively [22]; [23]; [24]. Considering the close relationship between IR 64 Sub-1 and the three popular varieties, this will be such an advantage to implement MABC for developing new variety with multiple tolerances. Cisantana is high yielding variety with potential up to 7.8 ton/ha and tolerant to brown plant hopper BPH biotype 2 and 3 and bacterial leaf blight (BLB) strain III. Mekongga is a high yielding variety that also known to have good rice eating quality, resistant to BPH biotype 2 and 3, and BLB strain IV. Whereas Angke, aside of high yielding, this variety is also resistant to BLB strain III, IV and VIII [22]; [23]; [24]; [25]. Introduction Sub-1 locus into these varieties will produce submergence varietal with other beneficial traits, and referring to the development of Inpari 30 (Cihaerang Sub-1), the MABC with close relation of parents will reduce the background recovery and thus shorten the achievement of the new variety [9].

4. Conclusion
Molecular polymorphisms were found between IR 64 Sub-1 and three popular varieties, Cisantana, Angke, and Mekongga. Polymorphic markers were identified in 11 chromosomes except for chromosome 12. These markers can be utilized in the background selection to support marker assisted backcrossing for development of new varieties with multiple tolerance and other beneficial traits. Prior to the settlement of the MABC program, additional polymorphic markers should be provided to fill the gaps especially in the missing locus in chromosome 11 and 12.
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