Marker selection on F2 population of Setail and ARC 12596 crossing

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Abstract. Tungro is an important rice disease. Rice tungro disease can decrease rice production. Resistance variety can be applied in plant breeding activities. Furthermore, using tungro resistance varieties more effective and efficient to control tungro disease. But, using resistance varieties continuously can breakdown resistance level because this character has durability. Therefore, the breeding of tungro resistance varieties should be done continuously. Breeding of tungro resistance can be held by using parental which resistance variety of tungro cross is with parental which have a specific location and have liked by farmers. This combination has a purpose to get tungro resistance which has a specific location. After crossing, the next step is to select the filial. This step should be done to evaluate the filial of cross-pollination and to get early line tungro resistance for the next material selection. The selection can be held by conventional and in-conventional or mix of both. The best selection is using mix procedure between conventional and in-conventional. Conventional selection is based on the standard evaluation system for rice (SES) IRRI. An in-conventional technique using SSR because it has an accurate technique to get genotype selection and purity evaluation. The purpose of this research is to know which SSR marker have linkage with tungro resistance in filial of a cross between tungro resistance parental and specific location. There are 3 markers SSR which can differentiate Setail and ARC 12596 to tungro disease those are RM 314, RM 212, and RM 288. Marker RM 314 has a chance to be a tungro resistance linkage marker from ARC 12596.

1. Introduction
Tungro is an important rice disease. Tungro is caused by two different virus types, there are Rice tungro bacilliform virus (RTBV) and Rice tungro spherical virus (RTSV) which infected by green leafhopper (GLH) in a semi-persistent way [5]. Rice tungro disease can decrease rice production in South Asia and South-East Asia [5; 6]. There are three components to control tungro. The first component is using resistance variety and planting at the same time, another component is eradication inoculum in the area and the last component is rolling varieties and manages planting time. These components should be combined with environment management and insecticide application [13; 10]. Resistance variety can be applied in plant breeding activities. Furthermore, using tungro resistance varieties more effective and efficient to control tungro disease because it can decrease RTSV so green leafhopper can’t transmit RTBV, in other words, tungro resistance varieties will reduce disease intensity. But, using resistance varieties continuously can breakdown resistance level because this character has durability [4]. Therefore, the breeding of tungro resistance varieties should be done continuously.
Breeding of tungro resistance can be held by using parental which resistance variety of tungro cross is with parental which have a specific location and have liked by farmers. This combination has a purpose to get tungro resistance which has a specific location so that it can decrease tungro infection and can be rolling varieties in that place. The next step after the crossing is selection of the filial. This step should be done to evaluate the filial of cross-pollination and to get early line tungro resistance for the next material selection. The selection can be held by conventional and in-conventional or mix of both. A conventional selection has advantages there are cheap and don’t need a specific technique. But this technique has a disadvantage, such as the low accuracy of tungro existence if there is only contain RTSV in a plant. Because RTSV can’t show the symptom [5; 9]. RTSV only can be detected by using the in-conventional technique. Therefore, the filial selection needs to be held using a mixed procedure between conventional and in-conventional. The in-conventional technique will be using markers from previous research which is linkage with tungro resistance. From the several in-conventional techniques, SSR is an accurate technique to get genotype selection and purity evaluation [1]. The purpose of this research is to know which SSR marker have linkage with tungro resistance in filial of a cross between tungro resistance parental and specific location.

2. Materials and Methods

2.1. Plant Material
ARC 12596 (ARC) as resistant varieties was crossing with Setail (ST) as sticky rice susceptible with tungro disease. The crossing technique is successfully done so F1 is obtained from each crossing. A F1 population is planted in the greenhouse to get the F2 population.

2.2. Plant Inoculation
Fifty plants of F2 from crossing ST x ARC is planted and inoculated with tungro virus. The population and parental are sowing in two trays, for each parental needs ten plants. Inoculation will be executed in 14 – 20 days after planting. This step is using 140 GLH that have been acquired into the infected plant (tungro isolate from South Sulawesi) for 48 hours in bulk with assumption 2 GLH each test plant. Two vector insects per plant are allowed to eat healthy plants (F2) for 24 hours. Observation is held on 1 until 3 weeks after inoculation based on standard evaluation system for rice (SES) IRRI [7].

Score 1 = no symptom observed
Score 3 = 1 – 10 % height reduction, no distinct yellow to yellow-orange leaf discoloration.
Score 5 = 11 – 30 % height reduction, no distinct yellow to yellow-orange leaf discoloration
Score 7 = 31 – 50 % height reduction, with distinct yellow to yellow-orange leaf discoloration
Score 9 = more than 50% height reduction, with distinct yellow to yellow-orange leaf discoloration.

2.3. DNA Extraction
DNA extraction was conducted using the CTAB method [12] with modified without liquid nitrogen and phenol. Leaf sample 0.1 – 0.2 grams were taken from the third leaf blade for each F2 plant. Then cut into pieces and crushed with 800 µl CTAB buffer and 10 µl of 2-mercaptoethanol. The homogenized tissues incubated in 65°C water bath for 60 minutes then centrifuged for 5 minutes at a speed of 11000 rpm. The supernatant was then transferred to a microtube and an equal volume of chloroform:isoamyl-alcohol (24:1) was added and homogenized well with a vortex. Centrifuged the mixtures at 11000 rpm for 10 minutes and the supernatant was transferred into a new microtube. Cold isopropanol (0.8 volume supernatant) was added into the supernatant. After overnight incubation in cold storage, the mixtures were centrifuged at 11000 rpm for 10 minutes and the DNA pellet was retained. DNA pellet was washed with 500µl of cold 70% ethanol, centrifuged and air-dried before diluted into 50µl TE buffer as DNA stock.
2.4. Microsatellite Marker

The diluted stock of DNA so that it is equivalent to 10 ng as a working solution. SSR markers at 34 loci (Table 1.) were evaluated on their ability to differentiate resistant parents and susceptible parents. PCR amplification was conducted using the following reaction mixtures: 1 µl DNA; 6.25 µl PCR KAPA-2G FAST Ready Mix buffer; 0.5 µl of primers (each F and R); 0.25 µl MgCl and 4 µl free nuclease water. Amplification was conducted using the following steps: one cycle of pre-denaturation at 94 °C for 2 minutes; 35 cycles of amplification steps at 95 °C for 30 seconds (template denaturation), annealing temperature for 1 minutes, and 72 °C for 1 minutes (extension), and one cycle of final extension at 72 °C for 5 minutes.

| No | Primer | Primer sequence | No | Primer | Primer sequence |
|----|--------|----------------|----|--------|----------------|
| 1  | RM8    | F: CACGTGGCGTTAAAATACACGTT R: GCCCAAAAAACCTAACCCTG | 18 | RM52   | F: CTACCTGCCCGTGGAAGTT R: TGCTTACTGGTGAAGCTGG |
| 2  | RM84   | F: TAAGGGTCCATCCCAAGAGTG R: TGCAATATCGACTGACTAC | 19 | RM108  | F: TCTCTTGCGCCACACTGGCAC R: GTGCAACCACCACCCACCA |
| 3  | RM212  | F: ACCACCCGTACGCTACTCAGG R: ACCACATTTTTCCTTATTAG | 20 | RM156  | F: GCCGCACCTCCTCCCTCTCTC R: CCTGCCGAGCCCTTGGAGTG |
| 4  | RM231  | F: CACGATATTACCTTCGAGGGTC R: CACTGCGATAGTTCGCA TTG | 21 | RM216  | F: GATGTGCCGATGTAAGAC R: TGTATAAAACCACACGGCC |
| 5  | RM232  | F: CCCGATATCCCTGATATTGC R: CCCGATATCCTCCGTGAAGCC | 22 | RM223  | F: GAGTGAGCTTGGGCTGAAAC R: GAAGGCAAGCTTTCGAGTC |
| 6  | RM273  | F: GGAGCGTGGTGGTTACAC T: GTTCTCTCCGTGAAGCC | 23 | RM230  | F: GCCAGACCGTGATGGTCC T: CACCGCAGTCACTTTTCAG |
| 7  | RM314  | F: CTGATTCAGACACTTTTCGAG R: AACATTCCACACACACGC | 24 | RM245  | F: ATCGGCCGACGTGATAGAC R: CTGAGATTTAATATCTT |
| 8  | RM324  | F: CTGATTCAGACACTTTTCGAG R: AACATTCCACACACACGC | 25 | RM341  | F: CAGAAAAACCTCAATCCGACG R: CTCTCCCGACACCTCA |
| 9  | RM343  | F: CCACGAAACCTTTTCGAC T: GTGATGATGGTTTCGAGG | 26 | RM409  | F: CGGTCTTCTTGCTTAAGGAATTC R: GGGGTGTGGTGGCTGG |
| 10 | RM431  | F: TCTGTACGGTAAAGAGTTG R: AGAGAAAACCTTTACGCTAC | 27 | RM427  | F: TCACTAGCTTCCCTCCCTGAC R: TGATGAGCTTGGTCCG |
| 11 | RM455  | F: AACAACCCACACCTTTTCGCTC R: AGAGAAAACCTTTACGCTAC | 28 | RM20   | F: ATCTTGTGCTCCCTCGAGTCAT R: GAAACAGAGGCACACCATTCACAT |
| 12 | RM505  | F: AGAGTTTAGATGCGCGTGTG R: GATTTTGCGGATCTTTACAGC | 29 | RM177  | F: CCCCCCCGACAGAAGCGCCAG AGG R: GATGCGAAGAGTGAGTCGG CC |
| 13 | RM507  | F: CTTAGCTCCAGCGAGAATG R: CTCCAGCTCCATCAGTCG | 30 | RM288  | F: CGGTACGTTCAAGCTCTTG R: AGCTAGGGAGGCTGGAGGAG |
| 14 | RM509  | F: TAGTGAGGGTGAGGGAAGAG R: AGAGCCTTAACAATCTTCAT | 31 | RM309  | F: GTAGATACGCACCTTGCTTG R: AGAGGGCCTCCCTTGGAGG |
| 15 | RM518  | F: CTCTTACCTCTCTCACTAGG R: ATCCATAGGCGAAAGCAAC | 32 | RM457  | F: CTCTCAGGATGCCCTTCTCTAG R: AGCTTAGATCTCAGAAGG |
| 16 | RM527  | F: GCCTGATCTGAAAATACCGG R: TGCAACAGGTTGGCAGT | 33 | RM491  | F: CATGAGTGCAGCGCATGTCAGG R: CTTCTCCCTCCAAATCTCT |
| 17 | RM574  | F: GCCTGATCTGAAAATACCGG R: TGCAACAGGTTGGCAGT | 34 | RM332  | F: GCAGAGGGCGAAAGGGAG T: RAGCATGAGTGCAGCGCATGTCAGG |

Table 1. List of SSR markers used
The generated SSR markers were separated using 8% polyacrylamide gel electrophoresis (PAGE) using TBE 1X buffer at 110 volts for 60 minutes and stained using silver staining [2]. The electropherogram was visualized over the light table. Regression analysis was performed using the Excel program

3. Results and Discussion

3.1 Scoring of Tungro

Of the 50 F2 seeds sown, only 44 plants were successfully grown. Scoring results showed a resistant score of 1 and 3, respectively 26 and 8 plants, a moderate score (score 5) there were 2 plants, and a susceptible score (scores 7 and 9) respectively 2 and 6 plants. Resistant plants have inherited several virus-resistant genes from ARC 12596 parents that can inhibit the development of RTBV and two recessive genes that control resistance to RTSV [11]. The tungro virus-resistant gene works by suppressing the occurrence of infection, inhibiting the process of replication and spread of the virus and reducing the accumulation of virus particles by inhibiting the assembly and stability of viruses [8]. Individuals who have a score of 1, 3 and 5 (resistant and moderate) can be used as material for further selection.

3.2 Microsatellite

DNA was isolated from 10 susceptible parents, 10 resistant parents and 44 F2 plants. 34 pairs of SSR markers have been selected through analysis of polymorphism in each parent DNA and 3 SSR markers can distinguish between resistant parents and susceptible parents, namely RM314, RM212, and RM288 (pic 1). The selected markers are then re-selected through PCR analysis to determine the allele of F2 to find out the presence of specific tungro resistant alleles.

![Figure 1](image1.png)

Figure 1. Amplified DNA bands from 3 SSR markers that have been able to distinguish resistant and susceptible parents

From those three primers, the primer was chosen again which has a specific allele that could determine F2 plants were infected by tungro with plants that were not infected by tungro. The selected primer is RM314. Figure 2 shows that the RM314 marker has three alleles. F2 inherits the allele from one of the two parents. The resistant parent has an allele around 122 bp while the susceptible parent has two alleles, 135 and 109 bp. Almost all plants with a score of 1 and 3 have the same allele with resistant parents (122 bp), as well as plants with a score of 7 and 9 having the same allele as susceptible parents (109 bp).

Otherwise, markers RM288 and RM212, allele in infected plants is not the same with allele Tungro resistance parent. RM288 has three alleles with a range of 134 to 239 bp. Susceptible plants have 239 bp alleles while resistance plants have 134 bp alleles. At F2 with a score of 1 and 3 alleles follow a resistant parent pattern, whereas in plants with susceptible scores 7 their alleles also follow a resistant parent pattern. The same at RM212, the plant scores were resistant and susceptible to having the same allele following the pattern of the susceptible parent, around 118 bp.
Figure 2. DNA amplification of RM314, RM288 and RM212 in F2 and their parents

From pic 2 indicates that there is an opportunity for markers RM314 to become a linked marker of resistance to the tungro virus from ARC parents based on the resulting tungro symptoms. In the study [3] it was also seen that the primers used as markers were primers who could distinguish between resistant parent (TW16, Utri Merah derivates) and susceptibility (Unkwang) and their F2. Regression analysis was performed for each primer. The result of R square from RM314, RM288, and RM212 are 0.44533, 0.21676, and 0.09852 respectively. The highest value is indicated by RM314. It could be said that RM314 can become a linked marker to differentiate susceptible parent and resistant one. RM314 also can differentiate lines which have different score of tungro.

4. Conclusion
RM 314 can become a linked marker of filial of a cross between ARC 12596 and Setail, and also can differentiate lines which have a different score of tungro.

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