Biosaintifika 10 (1) (2018) 153-139

Biosaintifika
Journal of Biology & Biology Education

http://journal.unnes.ac.id/nju/index.php/biosaintifika

Analysis of The Open Reading Frame (ORF) 29-TrnC (GCA) Sequence to Detect *Indica* and *Japonica* Sub-species on Upland Rice in Situ Bagendit and Inbred Rice of Ciherang

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DOI: 10.15294/biosaintifika.v10i1.12626

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History Article

Received 8 January 2018
Approved 6 March 2018
Published 30 April 2018

Keywords

Ciherang rice; Indica-Japonica differentiation; ORF 29-TrnC (GCA); Situ Bagendit rice

Abstract

The identification and the characterization of genetic diversity of rice was the first step in the rice plant breeding program. This study aimed to detect *indica* or *japonica* sub-species on upland rice Situ Bagendit and inbred rice Ciherang using molecular markers ORF 29-TrnC (GCA) on the chloroplast genome. Rice was included to the *indica* sub-species if the 32 bp insertion on ORF 29-TrnC (GCA) sequence was found, on the contrary, if the deletion 32 bp on ORF 29-TrnC (GCA) was found then it was included to the *japonica* sub-species. DNA isolation was examined from the leaves of the rice plants, and then it tested quantitatively to determine the transparency and DNA concentration from the isolation results. PCR amplification was performed using a pair of primers CP2 and it was followed by agarose gel electrophoresis. The visualization of the DNA bands used the gel documentation. Sequencing of PCR products produced a long base 390 bp in Situ Bagendit rice and 390 bp in Ciherang rice. Analysis of the sequences showed that the insertions occurred throughout the 32 bp in Situ Bagendit rice and the insertions occurred throughout the 32 bp in Ciherang rice. The results showed that upland rice Situ Bagendit and inbred rice Ciherang were included in the *indica* sub-species. The knowledge of variety of genetics of rice can be used as bio-information in the plant breeding program. Further, the knowledge can be used to protect in genetic power source, the selection and the composing of superior varieties of rice which is tolerant with kinds of biotic and abiotic factor.

How to Cite

Istiana, R., Kusumaningrum, H. P., & Ferniah, R. S. (2018). Analysis of The Open Reading Frame (ORF) 29-TrnC (GCA) Sequence to Detect *Indica* and *Japonica* Sub-species on Upland Rice in Situ Bagendit and Inbred Rice of Ciherang. *Biosaintifika: Journal of Biology & Biology Education*, 10(1), 153-139.

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p-ISSN 2085-191X
e-ISSN 2338-7610

INTRODUCTION

Rice cultivated in Asia is classified into two main sub-species based on the differences in morphology, physiology, biochemistry and molecular namely *Oryza sativa indica* and *Oryza sativa japonica* (Purwono & Purnamawati, 2009). In Indonesia, rice is the most dominant farming plant consumed by the public. The growing need for rice demanded the increasing rice productivity. Increasing rice productivity can be pursued by the assembly of the rice varieties with high yielding and resistant to diseases through the rice plant breeding program. This program can be done by conventional or biotechnology.

The identification and the characterization of genetic diversity on rice are important because it is the initial step in the rice plant breeding program (Nasir, 2001). One of them is to know the rice sub-species on *indica* or *japonica* type. Grouping the rice into *indica* or *japonica* sub-species is beneficial to map out the rice so that the plant breeders can be helped before doing whether the conventional crosses or genetic engineering. The cultivar IR8, as an example, is the result of crosses *japonica* cultivar "Deegeowoogen" with an *indica* cultivar "Peta" (BBPTP, 2010). Both sub-species can mutually fertilize but the percentage of success is low. It is because the *japonica* sub-species is more appropriate to cross with the *japonica* sub-species than others. On the contrary, the *indica* sub-species is also more successful to be crossed with the *indica* sub-species. Therefore, it is important to know the knowledge about the *indica* and *japonica* rice sub-species.

Rice sub-species grouping has been carried out not only through the morphology and anatomy approaches (Irawan & Purbayanti, 2008) but also a molecular approach (Haryanti e*t al.,* 2013). One of the effective molecular markers to classify the rice into the *indica* or *japonica* sub-species is a fragment of ORF 29-TrnC (GCA). The Open Reading Frame (ORF) 29 is the coding region and the TrnC (GCA) is a non-coding region of the chloroplast genome. The insertions throughout the 32 bp on the area of ORF 29-TrnC (GCA) was found in *indica* sub-species. On the contrary, the deletions throughout the 32 bp in *japonica* sub-species.

This study aimed to detect the *indica* and *japonica* sub-species on upland rice in Situ Bagendit and inbred rice of Ciherang. The results of this study were expected to complement the results of the characterization and the grouping in Situ Bagendit rice and Ciherang rice based on the morphology and the anatomy characters.

METHODS

The sample used in this research was the leaves of the upland rice in Situ Bagendit and inbred rice of Ciherang. Two comparative sequence of ORF 29-TrnC (GCA) of NCBI (National Center for Biotechnology) which was used is the *Oryza sativa indica* (AB983764.1) and *Oryza sativa japonica* (NC001320.1).

DNA isolation of chloroplasts was performed using the Doyle & Doyle method (1987) that has been modified. The leaves sample were weighed as much as 0,15 g then crushed on the gel ice. Pulverized leaves were put into the microtube and added the warm 200 µl buffer CTAB-2 ME then it was incubated in 65^0C for 2 hours. The sample was centrifuged 8500 rpm for 10 minutes to form 2 phases. The supernatant was taken and then added the chloroform: IAA (24:1) with a volume similar to the amount of supernatant (1:1). The sample homogenized with the vortex for ± a minute then it was centrifuged 8500 rpm for 10 minutes.The supernatant was taken and added the isopropanol cold (-20^0C) with similar volume to the amount of supernatant (1:1) and it was incubated 20^0C overnight. The samples were centrifuged 8500 rpm for 5 minutes, the supernatant was discarded and taken its pellet. The pellet was then added ethanol 80% and centrifuged 8500 rpm for 5 minutes. Ethanol was thrown away then the pellet was wind dried overnight. DNA samples were subsequently stored in the refrigerator in ddH_2O.

As much as 1 µl solution of ddH_2O was poured into the measuring hole of Nano-drop device, then it was closed. Next on the computer device, the read blank button was pressed. The rest of ddH_2o was cleaned by the tissue paper. DNA sample of 1 µl was inserted into the hole of the measuring Nano-drop device then read the sample. The measurement results in the form of DNA concentrations and the value of the purity of the DNA.

The amplification of regions of ORF 29-TrnC (GCA) that has been made referred to Li *et al.,* (2012) by using a pair of primers CP2 (5'-GCAGCCCAAGCGAGACT-3') as the forward primer and (5'-AAGGCTCGGCGATACTG-3') as the reverse primer. The amplification started with the pre-denaturation at a temperature of 95^0 C for 3 minutes, then 30 times repeated cycles consisted of denaturation 95^0C for 30 seconds, annealing at a temperature of 53^0C for 30 seconds and extension 72^0C for 50 seconds. The stage of the last extension carried out at a temperature of 72^0 C for 10 minutes and ter-

minated hold conditions at a temperature of 4^0C. PCR products were then performed electrophoresis using *agarose* 1% plus 1 µl of the gel read. The visualization of the DNA bands that appears was done with gel documentation.

The determination of DNA base sequence was made by the molecular biology company 1st BASE Malaysia. The results of sequencing between primer forward and reverse carried out the *contig* or the merger using the program Chromas-Pro to get a complete and good of nucleotides sequence. The sequence was blasted in the NCBI to get a comparative sequence of ORF 29-TrnC (GCA) of the *indica* and *japonica* sub-species. The results of sequencing and the sequence of the NCBI were alignment using the *ClustalX* program integrated into the Bioedit program to determine the homology and the sequence differences in order to detect an *indica* or *japonica* type. The molecular phylogenetic analysis is begun by alignment the sequensing results in the BLAST program on the NCBI site to get comparative sequence. The sequences have been obtained then aligned using the *ClustalW* program integrated into the MEGA 7.0 program. The phylogenetic tree reconstruction is formed based on the *Neighbor-Joining Tree* method

RESULT AND DISCUSSION

The DNA isolation results were analyzed quantitatively using the Nano-drop device. In general, the result of isolated DNA has a good concentration and purity (Table 1)

Table 1. The result of the DNA isolation for quantitative

Rice Varieties	DNA Concentrate (ng/µl)	The Value of The Absorbance (A260/A280)
SB[1]	1081.5	2.09
SB[2]	716.2	2.05
SB[3]	915.6	1.88
C[1]	295.3	2.08
C[2]	369	2.07
C[3]	644.5	2.04

Description. SB: Situ Bagendit, C: Ciherang, figures 1,2,3 show the replay.

The purity value of the DNA isolation results can be seen by measuring the absorbance value A260/A280. DNA with a good purity has an absorbance value of 1.8 to 2.0. If the value is more than that (>2,0), it is indicated protein contamination. On the contrary, if it is less than that (<1,8), it is indicated RNA contamination (Maftuchah & Zainuddin, 2015).

DNA isolation results then selected one to be amplified using PCR. Selected DNA in Situ Bagendit rice has a purity value of (1.88), while for Ciherang rice (2.04). The result of the amplification has a length between 300-400 bp (Figure 1).

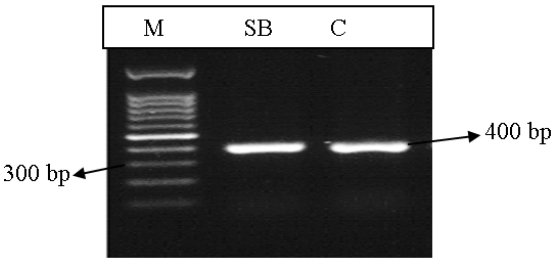

Figure 1. The electrophoresis result of PCR product. M: Marker, SB: Situ Bagendit, C: Ciherang

The result of sequencing the ORF 29-TrnC (GCA) in Situ Bagendit and Ciherang rice indicated the length of base that was not much different from the results of visualization of PCR products. The variation of the base length between the sequencing results and the sequences from NCBI was much different (Table 2 and Figure 2-5).

Table 2. The variation of the base length of ORF 29-TrnC (GCA)

ORF 29-TrnC (GCA)	The Length of The Base (bp)
Situ Bagendit	390
Ciherang	390
Oryza sativa indica (NCBI)	354
Oryza sativa japonica (NCBI)	322

>Situ Bagendit

GCAGCCCAAGCGAGACTTAC-
TATATCCATGTAAATTATGTCTCCTATTTC-
TATGAAGGAATTATTCTACTATTGAT-
GAATAATCATAGTAGAATCAAGGGTA-
CAGAGTCAAAAAGGGGTTCTGACCTA-
AAACTATGGATGAATCAGTTCAAAGAATT-
TACTCTTAACAAATTCTTAGAGTATTTCTG-
GTAGAATTTAACAAATTCTTAGAG-
TATTTCTGGTAGAATTGGGGAGCATTAAG-
TATAAATATGATACATAGCCCTTTCTTAT-
TAATAAAAGAATAAGGAAACGCTATCT-
CATCCCTATTGGTATCGGTTTGGGCCAC-
TACTGCTAAAACAAACCCCAGTTTGAG-
GAAAGAACGGTGGGTTCTCAAAATCCAG-
TATCGCCGAGC

Figure 2. The results of sequencing the ORF 29-TrnC (GCA) in Situ Bagendit rice

>Ciherang

GCAGCCCAAGCGAGACTTACTA-
TATCCATGTAAATTATGTCTCCTATTTC-
TATGAAGGAATTATTCTACTATTGAT-
GAATAATCATAGTAGAATCAAGGGTA-
CAGAGTCAAAAAGGGGTTCTGACCTAA-
AACTATGGATGAATCAGTTCAAAGAATT-
TACTCTTAACAAATTCTTAGAGTATT-
TCTGGTAGAATTTAACAAATTCTTAGAG-
TATTTCTGGTAGAATTGGGGAGCAT-
TAAGTATAAATATGATACATAGCCCTT-
TCTTATTAATAAAAGAATAAGGAAACGC-
TATCTCATCCCTATTGGTATCGGTTT-
GGGCCACTACTGCTAAAACAAACCC-
CAGTTTGAGGAAAGAACGGTGGGT-
TCTCAAAATCCAGTATCGCCGAGC

Figure 3. The results of sequencing the ORF 29-TrnC (GCA) in Ciherang rice

>*Oryza sativa indica* (NCBI)

AAAAAGGGGTTCTGACCTAAA-
ACTATGGATGAATCAGTTCAAAGAATT-
TACTCTTAACAAATTCTTAGAGTATT-
TCTGGTAGAATTTAACAAATTCTTAGAG-
TATTTCTGGTAGAATTGGGGAGCAT-
TAAGTATAAATATGATACATAGCCCTT-
TCTTATTAATAAAAGAATAAGGAAACGC-
TATCTCATCCCTATTGGTATCGGTTT-
GGGCCACTACTGCTAAAACAAACCC-
CAGTTTGAGGAAAGAACGGTGGGT-
TCTCAAAATCCAGTATCGCCGAGC-
CTTGTTATTCTCTTGCCCCAACTTAT-
GCGGGGTGCAAATTTGTCGATTTGGAT-
CAGTACTATAAGCCTAAGTA

Figure 4. The sequence of the ORF 29-TrnC (GCA) in *Oryza sativa indica* from NCBI

>*Oryza sativa japonica* (NCBI)

AAAAAGGGGTTCTGACCTAAGGC-
TATGGATGAATCAGTTCAAAGAATT-
TACTCTTAACAAATTCTTAGAGTATT-
TCTGGTAGAATTGGGGAGCATTAAGTA-
TAAATATGATACATAGCCCTTTCTTAT-
TAATAAAAGAATAAGGAAACGC-
TATCTCATCCCTATTGGTATCGGTTT-
GGGCCACTACTGCTAAAACAAACCC-
CAGTTTGAGGAAAGAACGGTGGGT-
TCTCAAAATCCAGTATCGCCGAGC-
CTTGTTATTCTCTTGCCCCAACTTAT-
GCGGGGTGCAAATTTGTCGATTTGGAT-
CAGTACTATAAGCCTAAGTA

Figure 5. The sequence of the ORF 29-TrnC (GCA) in *Oryza sativa japonica* from NCBI

The result of sequencing Situ Bagendit and Ciherang Rice has a difference on the bases length with the sequence of *Oryza sativa indica* and *Oryza sativa japonica* of NCBI. Some researches of bases length sequences ORF 29-TrnC (GCA) had been done, such as Shimada and Sugiura (1991) stated that the base length of ORF 29-TrnC (GCA) were ranged from 413 bp, where-as Li *et al.,* (2012) stated that the base length of ORF 29-TrnC (GCA) were ranged from 514 bp. The variation of this base length was caused by the presence of these deletions-insertions and used differences primers that caused a difference in the base length.

The difference of primers use can be seen from the result of the sequence alignment as the result of sequencing with the the sequence of NCBI. The alignment result showed that the homology of bases order (based on the mark *) was begun from Adenine bases and so on, besides the sequence of ORF 29-TrnC (GCA) Situ Bagendit and Ciherang rice was started with Guanine bases and so on (Figure 6).

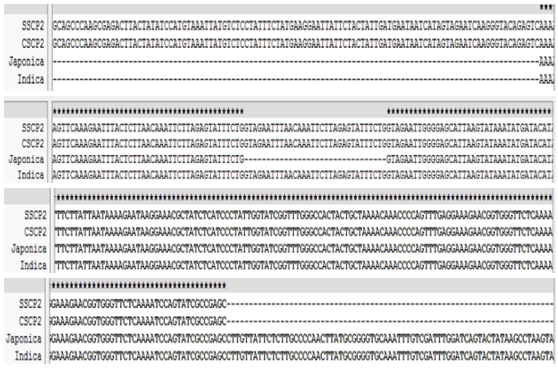

Figure 6. The result of the alignment of the upland rice Situ Bagendit and inbred rice Ciherang by sequence comparison from NCBI.Description. SSCP2: Situ Bagendit, CSCP2: Ciherang, Indica: *Oryza sativa indica* from NCBI, Japonica: *Oryza sativa japonica* from NCBI.

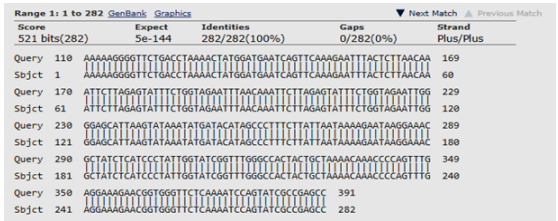

Figure 7. The result of the alignment of the upland rice Situ Bagendit and *Oryza sativa indica* by BLAST in NCBI. Description. Query: Situ Bagendit, Sbjct: *Oryza sativa indica*

This homology was supported by alignment between a sequence as one of the result of sequencing (ORF 29-TrnC (GCA) from Situ Bagendit rice) with the sequence of NCBI (ORF 29-TrnC (GCA) from *Oryza sativa indica*) on the

BLAST Program (Basic Local Alignment Search Tool) at NCBI Site.

The alignment of bases order with BLAST Program showed that the homology of bases order of sequence ORF 29-TrnC (GCA) Situ Bagendit Rice (query) with sequence ORF 29-TrnC (GCA) *Oryza sativa indica* (sbjct) was begun with the 110th bases on Situ Bagendit rice and the 1st bases of *Oryza sativa indica* (Figure 7). It showed that the order of bases 1 to 109 of Situ Bagendit rice was not homologous with *Oryza sativa indica.*

Then, the analysis done was by using sequence alignment as the result of sequencing and the sequence of NCBI with forward primer used. The result of alignment showed that forward primer was on the beginning of sequence ORF 29-TrnC (GCA) Situ Bagendit and Ciherang Rice (Figure 8). On the contrary, the result of forward primer alignment with sequence ORF 29-TrnC (GCA) of *Oryza sativa indica* and *Oryza sativa japonica* showed that forward primer was sparated and was on the central of sequence (Figure 9).

Forward primer has function to limit fragment which was in the beginning of target DNA that would be amplified. If sequence ORF 29-TrnC (GCA) of *Oryza sativa indica* and *Oryza sativa japonica* used CP2 primer, forward primer was in the beginning of sequence. It proved that primer used to amplify sequence ORF 29-TrnC (GCA) between Situ Bagendit and Ciherang Rice with *Oryza sativa indica* and *Oryza sativa japonica* of NCBI were different. The difference of primer caused the length of sequence ORF 29-TrnC (GCA) as the result of sequencing with sequence of NCBI different.

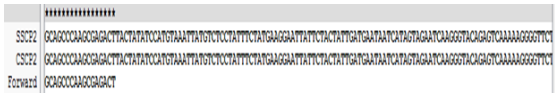

Figure 8. The result of the alignment of the upland rice Situ Bagendit and inbred rice Ciherang by forward primer. Description, SSCP2: Situ Bagendit, CSCP2: Ciherang, forward: primer CP2.

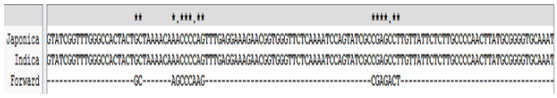

Figure 9. The result of the alignment of the sequence ORF 29-TrnC (GCA) from NCBI by forward primer. Description, Indica: *Oryza sativa indica* from NCBI, Japonica: *Oryza sativa japonica* from NCBI, forward: primer CP2.

The second difference of the bases length of the sequence of ORF 29-TrnC (GCA) was caused by bases insertion and deletion. The sequences of DNA in Situ Bagendit rice, Ciherang and *Oryza sativa indica* was found the insertion of 32 bp. Situ Bagendit rice, the insertions occurred at the 166-198 base. The insertions of Ciherang rice occurred on the 166-198 base, where-as in *Oryza sativa indica* from the NCBI, the insertions occurred at the 57-89 base (Figure 6). This is in accordance with Li *et al.,* (2012) who stated that the *indica* sub-species rice on ORF 29-TrnC (GCA) found that it has the insertion of 32 bp. The insertion was used as the focus to distinguish *indica* or *japonica* rice type. The results of the alignment showed that the upland rice of Situ Bagendit and inbred rice Ciherang was included in the group of *Oryza sativa* rice on *indica* sub-species.

So far, the process of grouping rice into subspecies *indica* and *japonica* still uses agronomy and morphology character (Sitaremi *et al*, 2013). Besides, this grouping system is known that is very influenced by environmental factor. Haryanti *et al,* (2013) uses DNA bands as the result of PCR amplification with CP2 primer to detect sub species of *japonica* and *indica*. By the existence of DNA bands as the result of PCR amplification so it could be clustered into *japonica* type, besides the disappearance of DNA bands as the result of PCR amplification could be clustered into *indica* type. However, this grouping process still had weaknesses. For example, the result of PCR amplification which was done by using CP2 primer on Situ Bagendit and Ciherang Rice also resulted DNA bands, but both of them belonged to *indica* type.

The analysis of sequence ORF 29-TrnC (GCA) gave more accurate result in grouping subspecies of rice because there was polymorphism in this area so that it could be distinguished between subspecies rice of *indica* and *japonica*. Dharmayanti (2011) believes that the use of DNA sequence in filogenetic study offers more accurate and faster data. Supporting this idea, Millah *et al,* (2012) also states that the analysis of genetic diversity by using molecular line such as microsatellite that helped showing diversity fast with high level of accuracy.

The next analysis is molecular phylogenetic analysis using ORF 29-TrnC (GCA) sequence. The moleculer phylogenetic analysis had purpose to know family's relationship among some species based on evolution's relationship using molecular markers. The molecular markers is used in phylogenetic analysis caused by DNA characteristic has known relative more consistent than morphology character (Hidayat & Pancoro, 2008). The phylogenetic analysis had purpose to

know about family's relationship among Situ Bagendit rice, Ciherang rice with some species rice from database in NCBI. Li *et al.,* (2012) said that polymorphisms in the chloroplast genome can be explained the evolutionary relationship between *O. sativa* and *O. rufipogon* (wild type) of Asian rice. Further, the polymorphism of ORF 29-TrnC (GCA) can be classified *O. sativa* into *indica* and *japonica* subspesies.

The phylogenetic tree formed consist of Situ Bagendit rice, Ciherang rice, 8 species rices that have relationship in one genus, one species that has relationship in one ordo and one species as out group. The phylogenetic tree reconstruction based on sekuen ORF 29-TrnC (GCA) were consist of two groups (Figure 10). The first group were consist of all spesies of *Oryza* with bootstraps score 100. The bootstraps score showed that high trust of branches formed. The first group had two branches. The first branch had bootstraps score 75 and the second branch had bootstraps score 40. The second branch separated from the first branch but still in the same group (Group I). Dharmayanti (2011) adds that sequences have near relationship can indentificate to take the same branch from the phylogenetic tree.

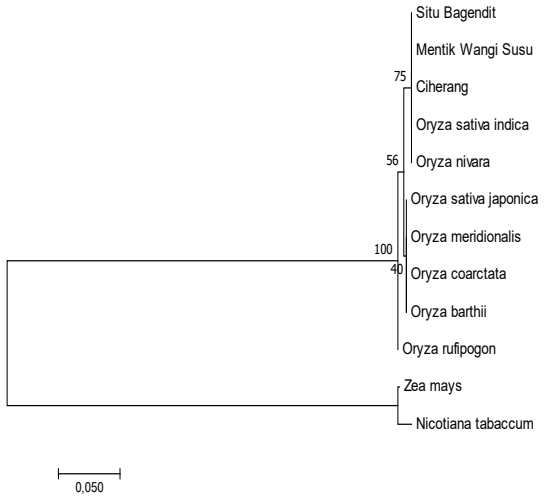

Figure 10. Phylogenetic tree of Situ Bagendit and Ciherang rice based on ORF 29-TrnC (GCA) sequence.

The first group showed that these rices have indication came from the same common ancestor (monophyletic) (Figure 10). The member of monophyletic group are assumed had the same genetic characteristic and biochemical (Hidayat *et al,* 2008). Situ Bagendit rice and Ciherang rice based on ORF 29-TrnC (GCA) were included into *Oryza sativa* subspecies *indica.* The phylogenetic analysis showed that Situ Bagendit rice, Ciherang rice, *O. sativa* subspecies *indica* (from NCBI) and *O. nivara* were in the same branch of the first group. It explained that these rice had near relationship. *Oryza rufipogon* (wild type of rice in Asian) located in separate place in the branch of *Oryza* group (Group I). Li *et al.,* (2012) stated that *O. rufipogon* based on ORF 29-TrnC (GCA) had an *indica/japonica* polymorphism as in *Oryza sativa*, but the evolution of *O. rufipogon* separated from *O. sativa*.

Situ Bagendit and Ciherang rice are examples of some kinds of local rice in Indonesia. Genetic power souce of local rice variety had a genetic superiority. Situ Bagendit rice was well known as one variety of upland rice that was tolerant of drought (BPPTP, 2010) and Ciherang rice was well known as one variety of irigated rice that was tolerant of the brown planthopper biotype 2 and the leaf blight of bacteria strain III and IV (BPPTP, 2010). The information of sub-spesies of this variety that could be a recommendation material to decide the potential variety to be developed better in plant breeding program. Silitonga (2004) adds that the use of genetic power source needs to be increased by using local varieties that has been characterized and evaluated to increase the genetic diversity of local variety that has been released.

CONCLUSION

The molecular marker of the chloroplast genome, namely the area of ORF 29-TrnC (GCA) can be used to classify rice sub-species into the *indica* or *japonica* type. Analysis of the ORF 29-TrnC (GCA) sequence showed that upland rice Situ Bagendit and inbred rice Ciherang were included in the *indica* sub-species.

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