Application of \textit{rps16} Intron and \textit{trnL-trnF} Intergenic Spacer Sequences to Identify Rengas Clone Riau

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\textbf{Abstract}

Rengas clone Riau has been identified using morphological characters and molecular technique with a \textit{psbA-trnH} intergenic spacer, however, this method can only determine its taxonomic status at genus level, namely \textit{Gluta} sp. This study reports application two DNA barcodes, i.e. \textit{rps16} intron and \textit{trnL-trnF} intergenic spacer, to identify Rengas clone Riau. The methods included collection of the leaves from Kajui Lake, total DNA isolation, electrophoresis, PCR (polymerase chain reaction), gel purification and sequencing. The \textit{rps16} intron size was 659 bp and the \textit{trnL-trnF} intergenic spacer was 527 bp. The BLASTn analysis showed that sequences of the \textit{rps16} intron and the \textit{trnL-trnF} intergenic spacer of \textit{Gluta} sp clone Riau had 100\% similarity to those of \textit{G. renghas} deposited in GenBank. These results were supported by high max score, high total score, query cover = 100\%, and E-value = 0. The dendrograms also showed the closest relationship of \textit{Gluta} sp clone Riau with \textit{G. renghas} deposited in GenBank compared to other species of \textit{Gluta}. In conclusion, this study succeeded in identifying Rengas clone Riau as \textit{Gluta renghas} by using sequences of the \textit{rps16} intron and the \textit{trnL-trnF} intergenic spacer. A combination of DNA barcodes could be applied to identify various plants as long as the database for the DNA barcodes is available in public database such as GenBank.

\textbf{How to Cite}


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INTRODUCTION

Riau Province in Indonesia has some flood plains and one of them is Kajuik Lake which is located in Pelalawan Regency. This lake is the floodplain of Kampar river and plays an important role for the life of the floodplain ecosystem, especially for the Riau endemic fish namely selais. In the rainy season, fish migrate to the lake and use the sideline of the roots of the trees that grow in and around the lake for spawning, laying eggs, caring fries, and providing shelters to protect fries from predators. In addition, the trees also contribute by maintaining erosion, to maintain water quality, and provide nutrients to the lake. Rengas is one of the trees that are growing there (Elvyra & Yus, 2012; Roslim, 2017).

Rengas from Kajuik Lake has been identified by Elvyra & Yus (2012) using morphological characters of leaf and stem without flower and fruit and then they determined it as Gluta sp clone Riau. Roslim (2016) then continued the identification using a DNA barcode such as psbA-trnH intergenic spacer region and expected that the species name could soon be determined. Unfortunately, this effort did not succeed because the sequence database of the psbA-trnH intergenic spacer of genus Gluta had not reached that point yet.

Further analysis of the morphology characters shows that there is similarity between Gluta sp clone Riau with G. renghas (Fern, 2014). At that time, the DNA barcode sequences for Gluta renghas was available in GenBank is rps16 intron and trnL-trnF intergenic spacer (Roslim, 2017). Therefore, the next research to determine the identity of Gluta sp clone Riau would be better off if it uses those DNA barcode sequences.

The DNA barcode is a piece of short DNA (approximately 700 bp) which position is known to be in the genome of organism, in the nuclear or organelle (mitochondria and chloroplast), and is used to identify an organism. The technique is called DNA barcoding (Hebert et al., 2003). Basically, this technique is developed to assist and to put organism identification at ease. People who are not experts in the field of taxonomy – such as employees of quarantines of animals, plants, geneticists, ecologists, etc. - can identify or determine the taxonomic status of the observed organism. In addition, molecular identification can still execute broken and incomplete specimen or in the condition of which even taxonomists are not able to identify it (Hebert et al., 2003).

Since 2003, scientists has developed some DNA barcodes and they have deposited these barcodes in public database such as GenBank (National Center for Biotechnology Information’s GenBank - NCBI, GenBank), EMBL (the European Molecular Biology Laboratory), and BOLD (the Barcode of Life Data System) (Stoeckle, 2003; Hebert & Gregory, 2005). Two DNA barcodes, i. e. matK and rbcL, have been agreed as a standard 2-locus barcode for plant identification due to the quality of the sequence, the ability to recover, and the ability to discriminate plant species (CBOL Plant Working Group, 2009).

In addition, the other DNA barcode such as a rps16 intron and a trnL-trnF intergenic spacer has been developed. Unlike the matK and the rbcL, encode the functional protein, the rps16 intron and the trnL-trnF intergenic spacer are parts of plant chloroplast genome that do not encode the functional protein (Sugita & Sugiura, 1996; Shaw et al., 2007; Borsch & Quandt, 2009).

The rps16 intron is an intron of a ribosomal protein S16 gene. Intron is a region in a gene that is transcribed but not translated in other words intron is a type of a non coding region in a genome. Generally, the non coding region is more vary and easier to mutate than the coding region (Borsch & Quandt, 2009). Coding region is also called exon that is a region in a gene which is transcribed and translated into protein, not easier to mutate, and relative conserved. There are 12 genes encoding ribosomal protein small subunit in soybean (Glycine max) (Daniell et al., 2016). The rps16 intron has been used for phylogenetic study of Angiosperms (Shaw et al., 2007) and Urophyleae (Smedmark et al., 2008).

Quite similar to the rps16 intron, the trnL-trnF intergenic spacer region is also a non coding region but located between two genes, i.e. trnL (UAA) gene and trnF (GAA) gene. This region has higher rate mutation and variation than the coding region such as matK and rbcL (Borsch & Quandt, 2009). The trnL(UAA) is located between the trnF(GAA) and trnT(UGU) in the plant chloroplast genome. The lengths of the trnL(UAA) exon on tobacco, rice, and Marchantia, respectively, are 577 bp, 614 bp, and 389 bp (Taberlet et al., 1991). The lengths of the trnL-trnF intergenic spacer on tobacco, rice, and Marchantia, respectively, are 438 bp, 324 bp, and 158 bp (Taberlet et al., 1991). The the trnL-trnF intergenic spacer region has been used to discriminate the species in genus Lophophora (Adrienne et al., 2015), to determine a new species of Atropa phasis (Yurtseva et al., 2016), to analyze the phylogenetic relationships within Pandanaceae (Buerki et al., 2012) and ferns (de Groot et al., 2011), to identify the tea plants which are used to produce commercial
tea and to provide information about the varieties used to make tea (Lee et al., 2016), and to analyze diversity and phylogeny of Myrtaceae (Vasconcelos et al., 2017) and Cycas chenii (Yang et al., 2016), to explain the genetic diversity of allopolyploid wheatgrass Elymus fibrosus (Schrenk) Tzvelev which is a member of Poaceae: Triticeae that is caused by its origin (Wu et al., 2016).

Scientists agree that it would be better to use multilocus DNA barcodes for plant molecular identification (Fazekas et al., 2008; CBOL Plant Working Group, 2009). The hypervariable non coding region, like the rps16 intron and the trnL-trnF intergenic spacer, is easier and preferred to be used for identification and discovery of a new species (Kress et al., 2009; Adrienne et al., 2015; Yurtseva et al., 2016). Therefore, this study reports the application of 2 DNA barcodes, i.e. the rps16 intron and the trnL-trnF intergenic spacer, to identify Rengas clone Riau. This research will provide information that identification of plants can be performed using a combination of DNA barcodes.

METHODS

Plant material used in this study was fresh leaves of Gluta sp clone Riau that grows in and around Kajuik Lake located in Langgam, Pelahawan Regency, Riau Province, Indonesia. The primer pairs for amplification of the rps16 intron and the trnL-trnF intergenic spacer were designed based on both sequences available in GenBank (Tabel 1).

Total DNA was extracted from fresh leaves of Gluta sp clone Riau using DNeasy plant mini kit (Qiagen). 0.5 gram of leaves was weighed and cut with scissors. After that, the pieces were crushed using mortar and pestel in liquid Nitrogen into powder. The powder was then poured into 1.5 ml tube for the next step according to the manufacture instruction (Qiagen). The pellet was then diluted with 50 µl of TE (Tris EDTA pH 8.0) and stored at 4°C. The quality and the quantity of the total DNA were predicted using electrophoresis technique.

Amplification of the DNA regions was performed using PCR technique with the following components: 1X PCR buffer (plus Mg²⁺), 0.1 mM dNTPs, 2.4 µM primer forward, 2.4 µM primer reverse, 2 U enzim Dream Taq DNA polymerase (Thermo Scientific), 1 ng DNA total, and water until 50 µl. The PCR conditions are as follows: 5 minutes at 94 °C for 1 cycle followed by 45 seconds at 94 °C, 45 seconds at 47 °C, and 1 minute at 72 °C for 35 cycles, and ended with 1 cycle of post-PCR for 10 minutes at 72 °C.

Electrophoresis is a technique used to separate the DNA fragments on a porous matrix under the influence of an electrical field. In this study, the electrophoresis was conducted to predict the quality and the quantity of the total DNA and also to check the success rate of PCR. It was done on 1.2% agarose gel in 1X TBE buffer (Tris Borate EDTA pH 8.0) at 65 volts for 30 minutes. Afterwards, the gel was stained by immersion in 5 µg/ml of ethidium bromide solution for 5 minutes then soaked in water for 5 minutes. Visualization of the DNA bands on the gel was performed using a UV lamp transilluminator (WiseUv WUV-M20, Daihan Scientific) and then photographed using a digital camera (Olympus SP-500 UZ).

Gel purification and sequencing of the PCR products were conducted by 1st Base in Malaysia via PT Gentika Science Jakarta, Indonesia. The PCR primers were used for the bidirectional sequencing.

The nucleotide sequence was then aligned using BLASTn program (Basic Local Alignment Search Tool) at http://www.ncbi.nlm.nih.gov/BLAST (Altschul et al., 1997) to find the similarity to the sequences in the GenBank database. Software of MEGA version 6.06 (Build#: 6140226) (Molecular Evolutionary Genetics Analysis) (Tamura et al., 2013) was used to create a dendrogram by Kimura 2-parameter model and UPGMA (Unweighted Pair Group Method with Arithmetic mean) with 1000 bootstrap.

RESULTS AND DISCUSSION

The PCR products for the rps16 intron and the trnL-trnF intergenic spacer were approxima-
tely 650 bp and 500 bp, respectively (Figure 1). The bands on the gel were clear and feasible for sequencing purposes.

![Figure 1](image1.png)

**Figure 1.** The DNA band profiles of (1) the rps16 intron and (2) the trnL-trnF intergenic spacer. M = 1 kb DNA ladder (Thermo Scientific).

### Analysis of The rps16 Intron Sequence

The length of the rps16 intron sequence was 659 bp. The sequence had been registered in GenBank with the accession number KX365741 (Figure 2). The BLASTn analysis to the sequence showed that the rps16 intron sequence of Gluta sp clone Riau had 100% similarity to the G. renghas sequence deposited in GenBank. This result was supported by the high value of max score, total score, and query cover, and the low value of E-value (Table 2).

![Figure 2](image2.png)

**Figure 2.** The rps16 intron sequence of Gluta sp clone Riau.

#### Table 2. BLASTn result of the rps16 intron of Gluta sp clone Riau.

<table>
<thead>
<tr>
<th>Description</th>
<th>Max score</th>
<th>Total score</th>
<th>Query cover</th>
<th>E value</th>
<th>Ident</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gluta renghas</em> voucher Pell 806 (BKL) ribosomal protein S16 (rps16) gene, intron; chloroplast</td>
<td>1189</td>
<td>1259</td>
<td>100%</td>
<td>0.0</td>
<td>100%</td>
<td>KP055393.1</td>
</tr>
<tr>
<td><em>Gluta tavoyana</em> voucher Pell 1075 (NY) ribosomal protein S16 (rps16) gene, intron; chloroplast</td>
<td>1160</td>
<td>1230</td>
<td>100%</td>
<td>0.0</td>
<td>99%</td>
<td>KP055394.1</td>
</tr>
<tr>
<td><em>Gluta wallichii</em> isolate 51 rps16 gene, intron; chloroplast</td>
<td>1157</td>
<td>1221</td>
<td>100%</td>
<td>0.0</td>
<td>99%</td>
<td>AY594600.1</td>
</tr>
<tr>
<td><em>Gluta tourtour</em> voucher Randrianasolo 770 (MO) ribosomal protein S16 (rps16) gene, intron; chloroplast</td>
<td>1148</td>
<td>1218</td>
<td>100%</td>
<td>0.0</td>
<td>98%</td>
<td>KP055395.1</td>
</tr>
</tbody>
</table>

Analysis to the rps16 intron sequence demonstrated that there were 19 nucleotide variations on some species of Gluta compared to Gluta sp clone Riau. Those variations were caused by mutations, such as insertion, deletion, and substitution, whether transition substitution and transversion substitution. The mutations occurred on Gluta sp clone Riau were the same as *G. renghas* deposited in GenBank (Figure 3).

Dendrogram generated from the rps16 intron sequences also showed that Gluta sp clone Riau had the closest relationship (distance = 0) with *G. renghas* as compared to others (Figure 4). This result confirmed that the species name of Gluta sp clone Riau was *G. renghas*.

![Figure 3](image3.png)

**Figure 3.** Differences among species of Gluta based on the rps16 intron sequences. The numbers ordered low showed the the position of the base according to Gluta sp clone Riau.

![Figure 4](image4.png)

**Figure 4.** Dendrogram based on the rps16 intron sequences calculated using UPGMA method with 1000 bootstrap. G = Gluta.
tered in GenBank with the accession number was KX365742 (Figure 5). The BLASTn analysis showed that the \(\text{trnL-trnF}\) intergenic spacer sequence of \(\text{Gluta sp clone Riau}\) also had 100% similarity to \(\text{G. renghas}\). This result was supported by the values of BLASTn parameters (Table 3).

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### Table 3. BLASTn result of the \(\text{trnL-trnF}\) intergenic spacer sequence of \(\text{Gluta sp clone Riau}\).

<table>
<thead>
<tr>
<th>Description</th>
<th>Max score</th>
<th>Total score</th>
<th>Query cover</th>
<th>E value</th>
<th>Ident</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Gluta renghas} ) voucher Pell 806 (BKL) (\text{tRNA-Leu (trnL)}) gene and (\text{trnL-trnF}) intergenic spacer, partial sequence; chloroplast</td>
<td>951</td>
<td>951</td>
<td>100%</td>
<td>0.0</td>
<td>100%</td>
<td>KP055514.1</td>
</tr>
<tr>
<td>(\text{Gluta laccifera} ) isolate Quyen 08 (\text{tRNA-Leu (trnL)}) gene and (\text{trnL-trnF}) intergenic spacer, partial sequence; chloroplast</td>
<td>924</td>
<td>924</td>
<td>100%</td>
<td>0.0</td>
<td>99%</td>
<td>KY067412.1</td>
</tr>
<tr>
<td>(\text{Gluta wallichii} ) (\text{tRNA-Leu (trnL)}) gene and (\text{trnL-trnF}) intergenic spacer, partial sequence; chloroplast</td>
<td>910</td>
<td>910</td>
<td>100%</td>
<td>0.0</td>
<td>98%</td>
<td>AY594516.1</td>
</tr>
</tbody>
</table>

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Figure 5. The \(\text{trnL-trnF}\) intergenic spacer sequence of \(\text{Gluta sp clone Riau}\).

Analysis to the \(\text{trnL-trnF}\) intergenic spacer sequence demonstrated that there are 15 variations between species of \(\text{Gluta}\) observed and those variations were due to mutation, for example, insertion, deletion, and substitution. Mutations occurred on the sequence of \(\text{Gluta sp clone Riau}\) were the same as \(\text{G. renghas}\) (Figure 6).

Figure 6. Differences among species of \(\text{Gluta}\) based on the \(\text{trnL-trnF}\) intergenic spacer sequences. The numbers ordered low showed the position of the base according to \(\text{Gluta sp clone Riau}\).

Dendrogram created using the \(\text{trnL-trnF}\) intergenic spacer sequences also showed the close relationship of \(\text{Gluta sp clone Riau}\) with \(\text{G. renghas}\) than \(\text{G. laccifera}\) and \(\text{G. wallichii}\) (Figure 7).

Like the analysis results based on \(\text{rps16}\) intron sequence, the analysis using the \(\text{trnL-trnF}\) intergenic spacer also succeeded confirming the taxonomic status of \(\text{Gluta sp clone Riau}\) as \(\text{G. renghas}\).
DNA barcodes, namely matK and ITS sequences, and also BLASTn analysis. The parameters values obtained were max score = 937, E-value = 0.0, identity value = 100%, and query cover = 100% (Roslim et al., 2016a). This success was supported by the availability of the matK and the ITS sequences for Elaeocarpus floribundus in GenBank.

The identification using DNA barcoding does not work if the DNA barcode sequences for the organism of interest do not exist in a public database like GenBank (Will & Rubinoff, 2004). Moreover, if the DNA barcode sequences of the organism of interest do not match the sequences existed in the public database, there will be 2 possibilities. First, the organism is a known species but the DNA barcode database is not available in the public database. Second, the organism is a new species. If the choice falls on the second possibility, the morphological justification must be conducted by following the taxonomic rules to make a conclusion that the organism is a new species (Will & Rubinoff, 2004; DeSalle, 2006; Roslim et al., 2016b).

In this study, few mutations have occurred in both of the rps16 intron and the trnL-trnF intergenic spacer on some species of Gluta. Mutation is a change on the DNA such as insertion, deletion, substitution, translocation, and inversion. Mutations detected in both of the sequences are insertion, deletion, and substitution, wether transition and transversion. Mutation can cause variation between species and this is favorable for phylogenetic analysis and determination of organism identity (Kelchner, 2002; Smedmark et al., 2008). Ryzhkova et al. (2013) has analyzed the indels and substitution mutations on the rps16 intron sequence to identify 6 haplotypes on Solanum. In addition, due to variation caused by mutation on the trnL-trnF intergenic spacer, Adrienne et al. (2015) can identify a species in genus Lophophora. A new species of Atraphaxis is also determined based on the trnL-trnF intergenic spacer (Yurtseva et al., 2016).

The DNA barcoding technique is basically developed to assist and to facilitate in identifying an organism using the DNA barcode sequences. However, there are some considerations in using this technique for plant identification, namely: (1) prediction of the genus of the observed plant based on morphological characters; (2) determination of types of the DNA barcodes which will be used by examining and selecting 1 to 4 of the DNA barcodes related to the observed genus which amount is abundant in public database; (3) amplification of the DNA barcodes using universal primer or own designed primer based on the conserved region; (4) performance of the BLASTn analysis and conclusion based on the BLASTn parameters. If the sequence of the observed plant has similarity (with ident value = 100%) to sequences in GenBank database, the plant is the same; (5) the conclusion should be better verified with the morphological or other corroborating data.

CONCLUSIONS

This study has succeeded in applying sequences of the rps16 intron and the trnL-trnF intergenic spacer to identify Rengas clone Riau as Gluta renghas. This success is supported by the availability of those sequences in GenBank. Thus, the DNA sequences database availability is critical for plant molecular identification.

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