Isolation of Partial Housekeeping Genes on Tuntun Angin (*Elaeocarpus floribundus*)

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DOI: http://dx.doi.org/10.15294/biosaintifika.v11i2.19052

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Abstract

Some genes like *18S rRNA*, *26S rRNA*, elongation factor 1-alpha (*EF1α*), and beta-tubulin (*TUB*) are members of housekeeping genes group that are commonly used as internal control in gene expression study. This study aimed to isolate those four housekeeping genes of tuntun angin (*Elaeocarpus floribundus*). The research material included fresh leaves of *E. floribundus* that were picked up from Kajuik Lake in Riau Province and four primer pairs. The procedures consisted of total DNA isolation using Genomic DNA Mini Kit Plant (Geneaid), polymerase chain reaction (PCR), electrophoresis on 1% agarose gel, sequencing, and bioinformatic analysis. This study has been isolated *18S rRNA*, *26S rRNA*, *EF1α*, and *TUB* genes with the size of 422 bp, 922 bp, 856 bp, and 877 bp, respectively. The *EF1α* and *TUB* genes has never been reported in Elaeocarpaceae family. Thus, those partial DNA sequences are the first sequences reported from this species and can be used as reference genes in this plant after validation.

How to Cite

Roslim, D. I., Ashfira, A., Mutiarawati, D., Rosmeilinda, T. F., Aisyah, N., Herman, H., & Lestari, W. (2019). Isolation of Partial Housekeeping Genes on Tuntun Angin (*Elaeocarpus floribundus*). Biosaintifika: Journal of Biology & Biology Education, 11(2), 194-201.
INTRODUCTION

Housekeeping genes are a group of genes which are coding proteins responsible for basic cellular functions in organisms – such as cell structural components, translation, ubiquitination, and glycolysis – and their expressions were abundant and relatively stable at any developmental stages and tissues (Sinha et al., 2015). Some of housekeeping genes are the genes encoding actin (ACT), tubulin (TUB), ubiquitin (UBQ), ribosomal RNA (rRNA), elongation factor-1-alpha (EF1α), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), serine/threonine-protein phosphatase catalytic subunit (PP2A), aquaporin tonoplast intrinsic protein (TIP2), YT521-B-like protein family protein (YT521-B), and histone 3 (HIS3) (Basa et al., 2009; Lee et al., 2010; Ray & Johnson, 2014; Pabuayon et al., 2016).

Actin and tubulin proteins are components of the eukaryotic cell cytoskeleton. In plants, the genes encoding actin and tubulin proteins have many variants and are called gene family. They participate in movement of chromosomes during mitosis and meiosis, movement of organelles, and intracellular transport (McDowell et al., 1996; Dominguez & Holmes, 2011; Kandasamy et al., 2012; Rebouças et al., 2013). Arabidopsis thaliana has ten actin genes (McDowell et al., 1996) while Pinus taeda (Schwarzerova et al., 2010) and Physcomitrella patens usually have seven to eleven actin variants (Zhang et al., 2010). Meanwhile, there are up to eight isotypes of beta-tubulin in plants, for example, six beta-tubulin isotypes in Physcomitrella patens (Jost et al., 2004), twenty beta-tubulin genes in Saxifraga arbutifolia (Rao et al., 2016), twenty in Populus (Oakley et al., 2007), nine in Arabidopsis thaliana (Cheng et al., 2001), and eight in Oryza sativa (Yoshikawa et al., 2003).

Ubiquitin protein is involved in recycle of damage proteins or other cell components and the process is named ubiquitination (Pickart & Eddins, 2004). Ubiquitin gene in plant also consists of many isotypes. Ubiquitin gene family in Arabidopsis thaliana comprises 14 members that are grouped into three types such as polyubiquitin, ubiquitin-like, and ubiquitin extension genes. The UBQ3, UBQ4, UBQ10, UBQ11, and UBQ14 genes are included as polyubiquitin genes and UBQ7, UBQ8, UBQ9, and UBQ12 genes are included as ubiquitin like genes (Callis et al., 1995).

Ribosomal RNA and elongation factor-1-alpha are involved in translation (Sasikumar et al., 2012; Gantasala et al., 2013). Ribosome is composed of ribosomal RNA (rRNA) molecules and ribosomal proteins. In eukaryotic cell, the ribosome is 80S in size and consists of large subunit measuring 60S and small subunit measuring 40S. In large subunit, the rRNA molecule consists of three types, namely 26S/28S, 5.8S, and 5S. In small subunit, there is only one type namely 18S. Genes of 28S, 5.8S, and 18S are in one-unit transcript separated by internal transcribed spacer 1 and 2 (ITS1 and ITS2). The 18S rRNA molecule has an important role in the accuracy of the start codon reading during translation initiation. The 18S DNA sequence is more conserved than 26S/28S (Gillespie et al., 2006; Porter & Golding, 2012). Validation of 26S/28S and 18S genes has been done and they have already been used as reference genes in plants (Bao et al., 2016; Hou et al., 2017; Singh et al., 2018).

The elongation factor-1-alpha (EF1α) is a GTPase enzyme that catalyzes the efficiency and accuracy of the installation of certain tRNA into ribosome and the elongation of polypeptide chains during translation elongation stage (Negrutskii & El’skaya, 1998; Talapatra et al., 2002). The EF1α, βTUB, and UBI genes are accurate enough to normalize expression data in jute (Corchorus capsularis) (Niu et al., 2015).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein is a key enzyme in glycolysis in all organisms. The GAPDH enzyme catalyzes the catabolism of glyceraldehyde-3-phosphate (G3P) to 1,3-biphosphoglycerate with the help of NAD and inorganic phosphate. Twenty-two GAPDH genes have been identified in wheat plants (Triticum aestivum) and they are grouped into four types namely gapA/gapB, gapC, gapCp, and gapN (Zeng et al., 2016). The GAPDH genes are reference genes that are often used the most as an internal control in plants because its expression is stable compared to other housekeeping genes (Sirover 2011; Kozera & Rapacz, 2013).

Tuntun angin (Elaeocarpus floribundus B1) is a medicinal plant but not widely known yet. This plant has some fruits with the shape and size resemble to olive fruit (Olea europaea) (Bhowmick, 2011; Sircar & Mandal, 2017). In Indonesia, E. floribundus can be found at Ruteng Park of natural tourism, in East Nusa Tenggara (Setiadi, 2004) and Kajuik Lake in Riau. Riau local people use its fruit for cooking (Roslim et al., 2016).

Part of the plant’s stem will submerge in rainy season for a few months but this plant still survives. This situation indicates that this plant has adapted to flooding stress and thus has some tolerant genes related to the flooding stress and also other stresses. Gene expression study may be performed to investigate the genes. The study requires some genes as internal control for target gene expression data normalization and the genes are called reference genes (Gantasala et al., 2013; Wang et al., 2016).
The reference genes come from housekeeping genes groups because their expressions are abundant and stable at any developmental stages, tissues, and conditions. Previously, a housekeeping gene was isolated from *E. floribundus* such as partial actin gene (Roslim & Herman, 2017). However, for the purposes of gene expression studies, more than one reference gene are needed as internal control (Rebouças et al., 2013; Wang et al., 2017; Bao et al., 2016). Moreover, the most widely used housekeeping genes as internal control are 18S rRNA, 26S rRNA, *EF1α*, *GAPDH*, actin, and beta tubulin (*βTUB*) in plants like tea (*Camellia sinensis*), jute (*Corchorus capsularis*), and olive (*Olea europaea*) (Kozera & Rapacz, 2013; Ray & Johnson, 2014; Niu et al., 2015; Wang et al., 2017). Therefore, the objective of this research was to isolate the partial housekeeping genes in *E. floribundus* such as genes encoding 18S rRNA, 26S rRNA, *EF1α*, and *βTUB*. This research aims to provide information about some housekeeping genes of *E. floribundus* that have not been reported in the previous studies. The genes can be evaluated for possible using as internal control in gene expression study of this plant.

**METHODS**

Fresh leaves of tuntun angin (*Elaeocarpus floribundus* BI) for DNA extraction were collected from Kajuik Lake in Riau Province, Indonesia. The primer pairs used in this study are presented in Table 1.

Procedures in this study were performed based on Roslim et al. (2018). The fresh leaves were used to extract the total DNA by using Genomic DNA Mini Kit (Plant, Geneaid GP100). The total DNA obtained was then checked for quantity and quality using agarose gel electrophoresis. After that, polymerase chain reaction (PCR) was performed for amplification of partial genes of 18S rRNA, 26S rRNA, *EF1α*, and *βTUB*. The agarose gel electrophoresis was also done for checking of the existence of PCR products. Forty five microliters of the PCR products were sent to PT Genetika Science for purification and sequencing at 1st Base in Malaysia.

The DNA sequences were then analyzed using BioEdit version 7. Alignment using BLASTn was also conducted to determine the similarity to other sequences available in GenBank database. Dendrogram was then constructed using MEGA6 software. These sequences analysis were conducted following a procedure suggested by Roslim et al. (2018).

**RESULTS AND DISCUSSION**

**Profile of PCR Products**

The PCR products of 18S rRNA, 26S rRNA, *EF1α*, and *βTUB* genes were obtained with the size approximately of 400 bp, 1000 bp, 900 bp, and 900 bp, respectively (Figure 1). Those bands were thick and suitable for sequencing requirements.

**Analysis of Partial Housekeeping Gene Sequences**

The partial DNA sequences obtained in this study were 422 bp for 18S rRNA, 922 bp for 26S rRNA, 856 bp for *EF1α*, and 877 bp for *βTUB*.

| Primer   | 5'------------------------3' | Annealing Temperature (°C) | Region                  | References          |
|----------|-----------------------------|-----------------------------|-------------------------|---------------------|
| 18S_F    | CGCGCAAAATTACCCAATCCT-GACA | 55.0                        | 18S ribosomal RNA       | Gantasala et al. (2013) |
| 18S_R    | TCCCGAAGGCCAACGTAAATAG-GA  |                             |                         |                     |
| 26S_NL1_F| GCATATCAATAAGCGGAG-GAAAG   | 56.1                        | 26S ribosomal RNA       | Porter & Golding (2012) |
| 26S_LR5_R| ATCCTGAGGGAAACTTC          |                             | elongation factor 1-alpha | Roslim et al. (2018) |
| EF1α_F   | GACCTGAGAAATCGGACC         | 54.7                        |                         |                     |
| EF1α_R   | TGGTGACATCTCAGAGCTTT       |                             |                         |                     |
| TUB3-F   | TGCGGCAAGGGGCACCTAYAC      | 58.4                        | tubulin                 | Einax & Voigt (2003) |
| TUB4-R   | GCCTCAGTGACTCCATCCTGTC-CAT |                             |                         |                     |
Figure 1. The PCR products of (1) 18S rRNA, (2) 26S rRNA, (3) EF1α, and (4) βTUB genes on Elaeocarpus floribundus. (L1) 100 bp DNA Ladder and (L2) 1 kb DNA Ladder (Thermo Scientific).

These DNA sequences have already been registered to GenBank database (Figure 2). Based on data in GenBank, the complete coding sequence of those genes were approximately 1754 bp for 18S rRNA, 2801 bp for 26S rRNA, 1350 bp for EF1α, and 1745 bp for βTUB (Emrich et al., 2007).

| GenBank ID | Organism            | Gene             | Length (bp) |
|------------|---------------------|------------------|-------------|
| M850498    | Elaeocarpus floribundus | 18S rRNA        | 1754        |
| M850499    | Elaeocarpus floribundus | 26S rRNA        | 2801        |
| M850501    | Elaeocarpus floribundus | EF1α             | 1350        |
| M850502    | Elaeocarpus floribundus | βTUB             | 1745        |

BLASTn analysis shows that the four sequences of *E. floribundus* have approximately 75.39%-99.04% similarity to the sequences deposited in GenBank database. The *E. floribundus* 18S rRNA and 26S rRNA genes have a higher similarity than EF1α and βTUB genes (Table 2). The result shows that the four genes are relatively conserved between species. The conserved genes are genes that have the same function in all organisms and the genes which involved in any biological processes, cellular localization, and molecular functions (Jayaswal et al., 2017).

Figure 2. The partial sequences of 18S rRNA, 26S rRNA, EF1α, and βTUB genes on Elaeocarpus floribundus.
exon (Rogozin et al., 2005; Zhu et al., 2009). Such conditions cause the EF1α to be able to be used as a marker to determine DNA contamination in total cDNA in addition to its potential as internal control in gene expression studies. For this purpose, PCR using DNA or cDNA molecule as template was performed separately and after that the sizes of the PCR products from the two templates were compared (Hannum et al., 2010). In this case, the PCR product from DNA template around 850 bp in length and cDNA template will produce a shorter PCR product which is about 750 bp in length. Previously, one housekeeping gene of E. floribundus was isolated, namely actin gene. The actin gene also contains two exons and one intron intron (Roslim & Herman, 2017) like the EF1α obtained in this study. Hence, in addition to the EF1α, the actin gene may also be used as DNA contaminant marker in total cDNA of E. floribundus. Housekeeping genes isolated from E. floribundus must be evaluated further to determine which one is appropriate as the reference gene. One of the validation processes can be done using the quantitative real time PCR (qRT-PCR) technique and the statistical analysis using geNorm, NormFinder, BestKeeper, and Rank Aggreg (Andersen et al., 2004; Pfaffl et al., 2004; Pihur et al., 2009; Bao et al., 2016). Some housekeeping genes have been validated using qRT-PCR technique in plants such as poplar (Basa et al., 2009), eggplant (Gantasala et al., 2013), rice (Pabuayon et al., 2016) and pigeonpea (Sinha et al., 2015). It is very crucial to select and validate the housekeeping gene for each treatment because it can thus reduce possible errors and contamination during extraction and manipulation of mRNA or cDNA (Rebouças et al., 2013).

The four housekeeping genes such as 18S rRNA, 26S rRNA, elongation factor 1-alpha (EF1α), and beta tubulin (βTUB) obtained in this study are the first reported from E. floribundus. After selection and validation, they can be used as internal controls in gene expression analysis in E. floribundus. For example, the study of gene expression related to flooding stress uses these genes as internal controls.

### Table 2. BLASTn result of the four housekeeping genes of tuntun angin (Elaeocarpus floribundus).

| Species                | Family              | Identity (%) | Accession   |
|------------------------|---------------------|--------------|-------------|
| 18S rRNA               |                     |              |             |
| Elaeocarpus sphaericus | Elaeocarpaceae      | 99.04        | GU476421.1  |
| Sloanea latifolia      | Elaeocarpaceae      | 99.04        | U42826.1    |
| Aristotelia serrata    | Elaeocarpaceae      | 98.80        | GU476422.1  |
| Elaeocarpus hookerianus| Elaeocarpaceae      | 98.80        | GU476420.1  |
| Brunellia acutangula   | Brunelliaceae       | 98.80        | FJ669718.1  |
| 26S rRNA               |                     |              |             |
| Elaeocarpus sphaericus | Elaeocarpaceae      | 98.68        | AY177422.1  |
| Crinodendron patagua   | Elaeocarpaceae      | 98.23        | AY935811.1  |
| Sloanea berteroana     | Elaeocarpaceae      | 98.12        | AF479126.1  |
| Eucryphia lucida       | Cunoniaceae         | 97.25        | AF036494.1  |
| Perrottetia longistylis | Dipentodontaceae    | 96.47        | AY935805.1  |
| Elongation Factor-1-alpha (EF1α) |  |  |             |
| Hedera helix           | Araliaceae          | 95.16        | KU942512.1  |
| Aralia elata           | Araliaceae          | 94.89        | JX067859.1  |
| Betula luminumfera     | Betulaceae          | 94.09        | KP245811.1  |
| Panax notoginseng      | Araliaceae          | 93.82        | KF815708.1  |
| Citrus maxima          | Aurantioideae       | 93.82        | JQ353767.1  |
| Beta-Tubulin (TUB)     |                     |              |             |
| Hordeum vulgare subsp. vulgare | Triticaceae     | 77.10        | AM502856.1  |
| Zea mays               | Andropogoneae       | 76.46        | NM_001348079.1 |
| Triticum aestivum      | Triticaceae         | 75.95        | U76744.1    |
| Alopecurus aequalis    | Poeae               | 75.87        | MH450177.1  |
| Oryza sativa           | Oryzae              | 75.39        | AB104732.1  |
so that the expression level can be trusted. Finally, the mechanism underlying this plant’s tolerance to flooding stress can be understood.

CONCLUSION

Four housekeeping genes such as 18S rRNA, 26S rRNA, elongation factor 1 alpha (EF1α), and beta tubulin (βTUB) were isolated from *tuntun angin* (*E. floribundus*) and these are the first genes reported from *E. floribundus*. The sizes of these genes are 422 bp for 18S rRNA, 922 bp for 26S rRNA, 856 bp for EF1α, and 877 bp for βTUB. Then, the four genes should further be validated so they can be used as reference genes. One gene, the EF1α, can be used as a marker of DNA contamination in total cDNA in gene expression studies of *E. floribundus*.

ACKNOWLEDGMENTS

This research was fully sponsored by a 2019 grant from Directorate General of Research and Technology and Higher Education, Republic of Indonesia under ‘Penelitian Dasar Unggulan Perguruan Tinggi’ with the contract number 756/UN.19.5.1.3/PT.01.03/2019.

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