

# CHARACTERIZATION OF 18S RIBOSOMAL RNA FRAGMENT FROM *Solanum tuberosum* L. var. *Granola* POTATO

YUNIOR WILLIAM SUSANTO<sup>1</sup>, HERMIN PANCASAKTI KUSUMANINGRUM<sup>1</sup>, SITI NURJANAH<sup>1</sup>,  
SRI RUSTINI<sup>2</sup>

<sup>1</sup>Biology Department, Faculty of Sciences and Mathematics, Universitas Diponegoro

<sup>2</sup>Balai Pengkajian Teknologi Pertanian (BPTP) Jawa Tengah

## ABSTRACT

Potato (*Solanum tuberosum* L.) is a prime horticultural commodity. One of the varieties of potato that widely cultivated in Indonesia is *Granola*. This study characterized the variety *Granola* based on the 18S sequences of rRNA gene fragment. The 18S sequences were used to distinguish the *Granola* and determine the differentiating characters from other *Solanum* based on those sequences data. The characterization was completed in three main steps including DNA isolation from potato leaf using Doyle & Doyle method, amplification of the 18S gene fragment, and DNA sequencing. The amplification of 18S gene fragment by a PCR method obtained 528 bp sequences. The BLAST search using NCBI web service confirmed that *Granola* potato has 99% matching sequence with *S. tuberosum*. The phylogenetic reconstruction further indicates the *S. tuberosum* var. *Granola* used in this study deeply nested with the reference sequence X67238.1, a potato from Europe.

KEY WORDS: *Granola*, 18S rRNA, phylogenetic, *Solanum tuberosum* L.

Corresponding author: HERMIN PANCASAKTI KUSUMANINGRUM | [herminsakti@gmail.com](mailto:herminsakti@gmail.com)

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## INTRODUCTION

*Solanum tuberosum* L, commonly known as potato, is a well-adapted plant that grows in several regions of Indonesia (Sutarno & Setyawan, 2015). This plant is a subtropical species that requires 16–21°C air temperature and 80–90% humidity for their growth (Williams *et al.*, 1993). In Indonesia potato is in high demand because they can substitute rice. Therefore, the potato is the leading horticultural commodities with increasing demand of 5% per year (Badan Ketahanan Pangan Kementerian Pertanian, 2013). The cultivation of potato requires an enhanced variety, one of which is *Granola*. This variety that originated from Germany is of interest for farmers because of the high production, short-lived, and adaptability in various condition (Kusmana & Basuki, 2004).

The Indonesian potato variety *Granola* has been published in 1993 including its morphological description in a variety release by a Decree of the Minister of Agriculture No. 444/1993. However, the information on the genetics of this variety including the genetic relationship with other varieties of *S. tuberosum* was insufficient. Therefore, molecular data is required in search for an improved strain with rapid growth time. According to Kawengian *et al.* (2016), molecular techniques can provide accurate genetic information because it is not affected by the environment. Along with the progress and development of agricultural technology, several molecular techniques have been developed to improve their potential. The molecular works include the use of gene sequence to identify the evolutionary relationship among a group of organism based on phylogenetic tree reconstruction for the breeding program purposes (Suwanto, 2011; Muzuni *et al.*, 2014).

Molecular techniques to track 18S rRNA sequences can be performed with the universal primer. In general, this technique uses universal primer based on the sequences of 18S ribosomal RNA of the eukaryotic plant. The 18S universal primer is designed based on a conserved sequence in the region of 18S ribosomal RNA, a relatively permanent part of the region.

This study was aimed to determine the genetic characteristic of the Indonesian potato variety *Granola* based on the 18S ribosomal RNA fragments using universal primer, and to determine its phylogenetic relationship with other variety of *S. tuberosum*. The result was expected to be used for a selective breeding program of the available varieties and cultivars of potato in Indonesia.

## METHODS

This study was conducted in the Integrated Laboratory, Universitas Diponegoro, Semarang, from February to April 2017. The healthy and fresh leaf samples were taken from one-month-old potato plant variety *Granola* (Runtunuwu *et al.*, 2011). The leaf samples from four individuals (G1, G2, G3, and G4) were collected in a plastic bag and kept in a coolbox for further analysis in the laboratory.

The leaf sample (0.34 g) was washed, dried, and sprayed with 70% alcohol. It was then crushed in a mortar using pestle on top of cold gel iceblock until smooth. CTAB+2ME solutions (3 ml) was added while the leaf sample crushed. The solution was then incubated at a temperature of 65°C for 30 minutes in a water bath, and homogenated every 10 minutes.

The solution was centrifuged at 8,000 rpm for 10 minutes. The supernatant was poured into a new centrifuge tube and added with CIA (24:1) based on 1:1 ratio. It was vortexed for one minute and centrifuged at 8,000 rpm for 10 minutes. The supernatant was stored in a new tube, then added with cold isopropanol (1:1), and incubated in a freezer overnight.

The solution was centrifuged next day at 8,000 rpm for 5 minutes. The supernatant was discharged, and the pellet washed with 500  $\mu$ l of 70% ethanol, and centrifuged for 5 minutes at 8,000 rpm. The pellet was dried for overnight followed by homogenization using ddH<sub>2</sub>O.

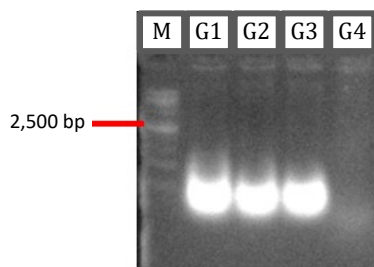
An electrophoresis technique was performed to test the DNA quality with 1% agarose gel at a 100-volt electrical current for 20 minutes. The electrophoresis was visualized using GelDoc. Quantitative DNA test was done by Thermo Scientific Nanodrop 2000 Spectrophotometer with as much as 1  $\mu$ l of DNA sample. DNA concentration was determined based on the absorbance value = A260, which equivalent to 50  $\mu$ g/ml DNA. DNA purity was determined based on the A260/280 = 1.8–2.0 (Sambrook *et al.*, 1989).

The 18S universal primer pair (Promega), 18S forward (5'-GTAGTCATATGCTTGTCT-3') and 18S reverse (5'-AGGGCAAGTGTGGTGCCAGC-3'), was used to target the 18S rRNA region. DNA amplification was started by making a mix solution for polymerase chain reaction (PCR) with a total of 25  $\mu$ l (2  $\mu$ l of the DNA template, 12.5  $\mu$ l KAPATaq Extra HotStart, 1.25  $\mu$ l 18S forward primer, 1.25  $\mu$ l 18S reverse primer, and 8  $\mu$ l ddH<sub>2</sub>O). The temperature cycle program used for amplification was initial denaturation temperature of 95°C for 3 minutes, final denaturation of 95°C for 15 seconds, primary adhesions (annealing) 52°C for 15 seconds, and elongation stage of 72°C for 1 minute. Amplification process was finished with a final elongation phase at a temperature of 72°C for 1 minute and stored on the final stage at a temperature of 4°C. The PCR process was run for 35 cycles.

Electrophoresis was performed by setting up 3  $\mu$ l PCR amplification products and 1 kb DNA Ladder of 250–10,000 bp (3  $\mu$ l DNA Ladder 1 kb mixed with 1  $\mu$ l loading dye), the mix was inserted into each well of a 1% agarose gel block and soaked in 1X TAE buffer. The running process was performed at 100 volts for 30 minutes. DNA bands were visualized using GelDoc. The ABI Prism 377 DNA Sequencer were used to sequence the target gene fragment. Phylogenetic analysis was done using a MEGA-6 computer application to align 18S rRNA sequences of Indonesian variety of *Granola* and other varieties of *S. tuberosum* obtained from NCBI website and to reconstruct the phylogenetic tree.

## RESULTS AND DISCUSSION

The qualitative test to identify and quantify the presence of DNA showed three out of four samples of *Granola* potato were successfully isolated visualized by the bright band in the agarose gel block (Figure 1).



**Figure 1.** The visualization of total DNA of the Indonesian potato variety *Granola* (M = DNA ladder, G1 = Sample #1, G2 = Sample #2, G3 = Sample #3, G4 = Sample #4).

The measurement of total DNA concentration and purity is presented in Table 1. Among the four

samples, the highest concentration was obtained from sample G1, and the lowest was sample G4.

**Table 1.** Total DNA quantification of the Indonesian potato variety *Granola* using Spectrophotometer

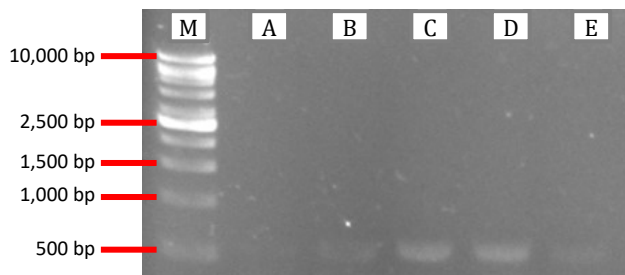
Sample	Concentration	A260	A280	A260/280
G1	8,337.2	166.745	77.407	2.15
G2	1,285.8	25.715	13.579	1.89 **
G3	793.1	15.863	7.865	2.02
G4	18.8	0.377	0.233	1.62

\*\* = DNA sample with the best value of purity

The four samples have DNA purity value of 1.62–2.15 ng/ $\mu$ l. According to Sambrook *et al.*, (1989) the purity value of DNA is between 1.8 and 2.0. The value less than 1.8 indicates phenol or protein contamination (Devereux & Wilkinson, 2004), while more than 2.0 indicates RNA contamination (Khosravinia *et al.*, 2007). In this study Sample G2 is having the best value of DNA purity with no indication of contamination. The DNA isolates in this study was obtained with the brownish pellets instead of a usually white pellet. The brown colour may be indicated by co-precipitation of polysaccharides and oxidation of phenolic compounds. Polyphenols have properties to be readily oxidized by air and bind to DNA at the time of isolation of DNA (Lodhi *et al.*, 1994).

The success of the DNA amplification is determined by the thermal cycler program, especially annealing temperature. According to Handoyo & Rudiretna (2001), annealing temperature can be determined based on melting temperature ( $T_m$ ) of the primer that is used between  $(T_m-5)^\circ\text{C}$  until  $(T_m+5)^\circ\text{C}$ , while theoretically the  $T_m$  of the primer can be calculated by using the formula of  $[2(A+T)+4(C+G)]$ . Therefore annealing temperatures ranges often used for PCR is about 50–60°C.

Determination of annealing temperature was conducted by optimization of PCR conditions to get the best DNA amplification result. Optimization of PCR conditions is performed using a gradient PCR on five different annealing temperatures, *i.e.*, 49, 50, 51, 52, and 53°C. In this study the annealing temperature at 52°C was the most optimum temperature for PCR *Granola* potato using an 18S universal primer. The visualization of PCR products indicated the amplifiable DNA size was about 500 bp (Figure 2).



**Figure 2.** The visualization of the 18S rRNA PCR product of the Indonesian *Granola* potato. (M = 1Kb DNA ladder, A = G2 MT 49°C, B = G2 MT 50°C, C = G2 MT 51°C, D = G2 MT 52°C, E = G2 MT 53°C).

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1 TACCTGGTTG ATCCTGCCAG TAGTCATATG CTGTCTCAA AGATTAAGCC ATGCATGTGT
18S-F . . . . .
541 GGATCCATTG GAGGGCAAGT CTGGTGCCAG CAGCCCGGT AATTCCAGCT CCAATAGCGT
18S-R . . . . . G . . . . .
741 CTTATCATTT AGAGGAAGGA GAAGTCGTAA CAAGGTTTCC GTAGGTGAAC CTGCGGAAGG
1801 ATCATTG
    
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**Figure 3.** Alignment of upper and lower sections of 18S universal primer against the 1,807 bp reference sample of *S. tuberosum* X67238.1 from NCBI.

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Query 8 GCATGTGT-AGTATG-AC-AATTCAGACTGTGAAACTGCGAATGGCTCATTAAATCAGTT 64
Sbjct 53 . . . . . A . . . . . A . A . . . . . 111

Query 65 ATAGTTTGTTTGATGGTATCTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGT 124
Sbjct 112 . . . . . 171

Query 125 GCAACAAACCCCGACTTCTGGAAGGGATGCATTTATTAGATAAAAAGGTCGACGCGGGCTC 184
Sbjct 172 . . . . . 231

Query 185 TGCCCGTTGCTGCGATGATTTCATGATAACTCGACGGATCGCACGGCCATCGTGCCGGCGA 244
Sbjct 232 . . . . . 291

Query 245 CGCATCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAGTGGCCTACCATGGT 304
Sbjct 292 . . . . . 351

Query 305 GGTGACGGGTGACGGAGAATTAGGGTTCGATTCGGAGAGGGAGCCTGAGAAAACGGCTAC 364
Sbjct 352 . . . . . 411

Query 365 CACATCCAAGGAAGGCAGCAGGCGCAAATACCCAATCCTGACACGGGGAGGTAGTGA 424
Sbjct 412 . . . . . 471

Query 425 CAATAAATAACAATACCGGGCTCTATGAGTCTGGTAATTGGAATGAGTACAATCTAAATC 484
Sbjct 472 . . . . . 531

Query 485 CCTTAACGAGGATCCATTGGAGGGCAAGTCGGGTGCCAGCA 525
Sbjct 532 . . . . . T . . . . . 572
    
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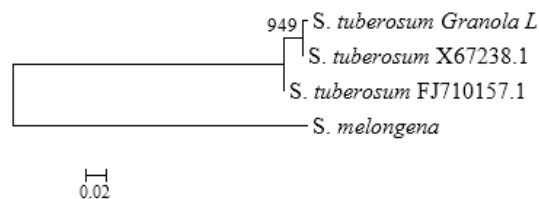
**Figure 4.** Homology analysis of the 18S rRNA sequences of Indonesian potato var. *Granola* with *S. tuberosum* X 67238.1 from NCBI (The query is the sequence of Indonesian potato var. *Granola*, while the Sbjct is the *S. tuberosum* X 67238.1 sequence)

The length of the sequence obtained in this study was 528 bp. The length of the DNA sample was estimated by the PCR product quantification around 500 bp. The result was also confirmed by the alignment of the 18S universal primer pair to the total length of 18S rRNA reference sequence of *S. tuberosum* obtained from NCBI website (Figure 3). The alignment showed the 18S forward primer attached to position 20–37 and the reverse primer attached to position 552–571 in the 1,807 bp length reference sequence of *S. tuberosum* X67238.1. This confirmed the 18S universal primer successfully amplified partial 18S rRNA region of the Indonesian *S. tuberosum* var. *Granola*.

The BLAST search in the NCBI website found two sequences within the species of *Solanum tuberosum* with approximately 99% matching sequence to Indonesian potato variety *Granola*, equal to 516 of 521 nucleotides were matched (Figure 4). The alignment with the highest matching sequence of *S. tuberosum* X67238.1 showed one substitution and four gaps. According to Dharmayanti (2011), the gap in multiple aligned sequences indicated the occurrence of indels of one or more characters in a sequence during evolution.

The phylogenetic analysis was conducted for Indonesian *Granola* potato with two highly matched sequences of *S. tuberosum* found using BLAST search, X67238.1, and FJ710157, with *S. melongena* selected as the outgroup. Indonesian potato variety *Granola* has a closer relationship with X67238.1 sample from Europe compared to FJ710157.1 sample from Asia

(Figure 6). The Indonesian *Granola* potato deeply nested with *S. tuberosum* X67238.1 with a bootstrap value of 949 or 94.9% confidence value. The value indicates the clade of Indonesian *Granola* potato and *S. tuberosum* X67238.1 from Europe form a relatively stable group. According to Felsenstein (1985), a clade with a confidence value of 95% or more and at least 90% is a stable clade.



**Figure 5** Phylogenetic tree of Indonesian *Granola* potato grouped with other samples of *Solanum tuberosum* with *Solanum melongena* selected as outgroup

The phylogenetic analysis confirmed the origin of Indonesian *Granola* potato from Germany (Europe) as described in the Decree of the Minister of Agriculture No. 444/Kpts/TP.240/6/1993. The phylogram shows the average of nucleotide substitutions per site is 0.02 for a given length. The evolutionary lineages indicated the Indonesian *Granola* potato share a common ancestor with *S. tuberosum* X67238.1 and *S. tuberosum* FJ710157.1 from genebank. In this study based on the partial 18S rRNA conserve region sequence we consider that Indonesian *Granola* potato was distinguishable although it has a highly similar trait with its close relative X67238.1 potato which is *S. tuberosum* from Europe. This similarity in the genetic

characteristics indicates that potato from Europe can be improved through the breeding program to be cultivated in Indonesia, referring to the considerable success and favorite variety *Granola* of Indonesian potato.

### CONCLUSION

The characterization of Indonesian potato variety *Granola* based on 18S rRNA fragment showed a 99% matching sequence to *S. tuberosum*. The phylogenetic reconstruction suggests the Indonesian *Granola* potato is deeply nested or closely related to *S. tuberosum* X67238.1 sample from Europe than FJ710157.1 sample from Asia.

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