ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Characterization of EF-1α Gene of *Conopomorpha Cramerella* Snell. As Cocoa Pod Borer.

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ABSTRACT

The aim of this study is to determine the character of the EF-1α gene of Conopomorpha camarilla as cocoa pod borer pests (CPBP), so it can be used as an ingredient of molecular information for early detection of CPBP by PCR. This study used a PCR method that begins with the isolation of genomic DNA of CPBP using CTAB method. Sequence analysis using NCBI programs, creation of phylogenetic tree using CLC sequences program, restriction enzyme mapping using NEB Cutter program, amino acid analysis using Expose program, and hydrophobicity analysis using Bio Edit program. The results showed that the character of a partial gene fragment EF-1α CPBP is 601 bp sizes; based on the percentage of similarity values, CPBP sample is the same species as C. camarilla because it has a value of 100% similarity with C. camarilla in GeneBank; restriction enzyme mapping showed 42 sites cutting; amino acid analysis produces 200 amino acids; hydrophobicity analysis showed partial gene EF-1α is the hydrophilic region dominance; and the results of construction phylogenetic based on nucleotides and amino acids sequences showed that CPBP are in the same clade with Conopomorpha camarilla. Based on these characters can be concluded that CPBP samples is included in Conopomorpha camarilla species. Specific primer used in this PCR can amplify specific DNA fragment of Conopomorpha camarilla so that it be applied as detection tools of Conopomorpha camarilla.

Keywords: Conopomorpha camarilla, CPBP, Elongation Factor 1 alpha gene, Cocoa

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ISSN: 0975-8585

INTRODUCTION

Cocoa is one of the commodities that have an important role for the economy in many countries, such as providers of employment, sources of income of farmers, and a source of foreign exchange. In Indonesia, production of cocoa (Theorem cacao L.) tends to increase every year, thus increasing the demand for domestic consumption and export. The increase in crop production can be done by implementing a program of the National Movement Cocoa include replanting, rehabilitation of the plant, and the expansion of planting area [1]. However, the main problem in increasing production is the quality and productivity of cocoa beans was low and is caused by the Cocoa Pod Borer Pests (CPBP), Conopomorpha cramerella. CPBP is a very destructive pest on cocoa plants and can reduce the production of up to 90% [2, 3, 4, and 5]. CPBP attacks cause the death of the placental tissue of the seeds so that the seeds cannot develop perfectly and then become sticky and cannot be harvested. The spread of CPBP in Indonesia reaches 348,000 ha [6] or 57% of the area of cocoa spread throughout the cocoa plantation area with varying severity of attacks between locations. The highest CPBP severity occurred in cocoa centers, such as in South Sulawesi, Southeast Sulawesi, Central Sulawesi and East Kalimantan. Therefore, the existence of CPBP can threaten the continuity of cocoa production in Indonesia.

One of the causes of CPBP attacks on crops high is the use of planting materials which are not free of CPBP. The use of infected planting material by pests will generate long-term huge losses [7]. One strategy in CPBP prevention is early pest control through the use of free CPBP planting materials. Early detection of diseases caused by CPBP can be performed using specific and sensitive detection techniques, i.e. PCR (Polymerase Chain Reaction) with specific primers.

The use of PCR method with specific primers could amplify specific regions contained on DNA so the method is very supportive in the development of detection techniques of CPBP. Prior to this detection method is applied, first performed design specific primers to amplify a specific DNA fragment of CPBP and characterize these fragments with bioinformatics approaches. The character was used to ensure that the fragment was part of the DNA of CPBP.

DNA fragments that can be characterized are DNA fragments CPBP provided in GeneBank. Currently, DNA fragments CPBP that is available in GeneBank are elongation factor-1 Alpha gene (EF-1α). EF-1α protein encoded by the gene EF-1α is one of four constituent subunits of eukaryotic translation elongation factor i.e., EF-1α, EF-1βγ, EF-2 and EF-3 highly abundant range of 3-10% of the total soluble proteins in the cell cytoplasm [8]. The protein has a key role in any process of translation in eukaryotic cells so that the DNA coding its protein possible to used as a template for designing specific primer that can be used in the identification of eukaryotic organisms with PCR method. EF-1α protein plays an important role in protein biosynthesis, catalyzes the binding of aminoacyl-tRNA to the A site of the ribosome with a GTP-dependent mechanisms [8].

The development of bioinformatics as a new science has made it easier to analyze a variety of data from research in the field of Molecular Biology. Program and data relating to Molecular Biology easily accessible from the internet, among others, to obtain nucleotide sequences stored in GenBank, analyze the level of similarity of nucleotide sequence with nucleotide sequence in GeneBank using Clustal-W, comparing a sequence with sequences stored in Genbank using Basic Local Alignment Search Tool (BLAST) program, specific primer design, and knowing the restriction enzyme sites within DNA strands [9]. This paper describes the characteristics of EF-1α gene from cocoa pod borer (C. cramerella) to the cocoa crop that will be used as a tool of pest detection by PCR.

MATERIALS AND METHODS

The materials used in this study are Conopomorpha camarilla Snell. (CPBP), primer, CTAB (Cetyl Trimetyl Ammonium Bromide) buffer, PCR kit, agarose, PCI (Phenol-Chlorofom-Is amyl Alcohol), quartz sand, ethidium bromide, 1X TAE (Tris-Acetic EDTA), 70% ethanol, ethanol absolute, dH_2O, RNase, Loading dye, TE buffer (Tris-HCl and EDTA), Marker (1 kb ladder).

Research can be done by identifying the molecular CPBP that had been taken from the study site. The identification process include the isolation of DNA, test the quality and quantity of DNA, primer design, as well as the amplification reaction, electrophoresis, DNA sequencing and sequence analysis [10].

Sampling

The sampling process is done by two methods namely by capturing the individual of CPBP directly and by way of incubating the infected cocoa pods and allowed to larvae in the fruit of the cocoa fruit out to the skin surface and form a pupa. At the time, the CPBP sample can be easily retrieved, identified and further done isolation of DNA.

Isolation of DNA

DNA of CPBP was isolated using the CTAB (Cetyl Trimetyl Ammonium Bromide) method [11]. Prior to isolation, lysis buffer prepared in advance according to the number of samples to be extracted. Samples were weighed as much as 0.1-0.2 g, and then crushed with the help of quartz sand. Samples were inserted into eppendorff 1.5 ml and added 600 mL of lysis buffer. Samples were incubated for 30 minutes at a temperature of 65^{o}C and inverted every 5 minutes. The samples were then put in ice for 5 minutes and then centrifuged at 10,000 rpm for 10 minutes. Supernatant is taken and put in a new eppendorff and added 1 x volume PCI (Phenol-Chlorofom-Is amyl Alcohol) that serves to separate contaminants such as proteins and organic compounds with DNA.

Furthermore, the suspension was centrifuged at 10,000 rpm, a temperature of 40^{o}C for 10 minutes. Supernatant is taken and transferred in 1.5 ml eppendorff then added with 0.1 volume of sodium acetate (NaOAc) 3 M pH 5.2 and 2 volume of absolute ethanol and then incubated for 2 hours and centrifuged for 20 min at 10,000 rpm 4^{o}C so that DNA pellet was obtained. Furthermore, the DNA pellet was washed with 0.5 ml of 70% ethanol, then dried and finally dissolved in 20 mL H_2O. To remove RNA, the solution was added 100 ug / ml RNase and incubated at 37 ^{o}C for 12 hours. DNA solution is then stored at a temperature of -4^{o}C.

Quality and Quantity Test of DNA

DNA quality can be measured by electrophoresis and spectrophotometer, while the quantity of DNA is measured by means of the isolated DNA spektrofotometer. Electrophoresis serves to determine whether the DNA intact or degraded. Spektrofotometer at a wavelength of 260 nm and 280 nm is used to determine whether DNA is pure or contaminated. Wavelength used to determine the DNA content using UV spectrophotometric is 260 nm, while to know the protein contaminant using UV spectrophotometric with a wavelength of 280 nm.

DNA quality is determined based on the value of the ratio of A260/A280 of around 1.8 - 2.0 [12]. DNA quantity is determined based on the assumption that 1 OD (Optical Density) = 50 ug/ml of double-stranded DNA, by the formula:

$$[DNA] = A260 \times 50 \text{ ug / ml} \times DF$$

[DNA] = Concentration of DNA
A260 = Absorbance at a wavelength of 260 nm
50 ug / ml = Constant for DNA

Primer Design

DNA sequences encoding EF-1α of several species closest obtained from gene data bank (GeneBank) aligned using Bio edit program. Areas that have a high homology (conserved region) can be used as specific primers in the PCR. Primers used to amplify specific fragments of genes encoding EF-1α are a pbk-F primer (5'-CTCTACTGA GCCCCCATACA-3') and pbk-R primer (5'-CTGGTCAAATCTCAACGGT-3').

PCR reaction and Electrophoresis

PCR reactions use DreamTaq Green PCR Master Mix (2X). The composition of the PCR reaction is 100 ng/µL DNA template 1 µL, 0.5 µM forward primer 0.5 µL, 0.5 µM reverse primer 0.5 µL, 2x Master Mix 5 µL and dH2O 3 µL. PCR reactions were performed by 30 cycles with the following conditions: pre PCR, for 5 minutes at a temperature of 94^{o}C; denaturation, for 1.5 minutes at a temperature of 94^{o}C; annealing for 1 min at 55^{o}C; extension, for 1.5 minutes at a temperature of 72^{o}C and post-PCR, for 5 minutes at a temperature of 72^{o}C.

ISSN: 0975-8585

The PCR reaction requires time for ± 2.30 hours. The results of amplification is then performed electrophoresis on a 1% agarose with a voltage of 100 volts and 80 mA for 30 minutes and then visualized on top of UV Tran illuminator then do a photo shoot with photoforesis.

DNA sequencing

DNA sequencing of amplification product using automated DNA sequence tool (automated DNA sequence ABI Prism 310, Perkin-Elmer). DNA sequencing is done by the Sanger method, using dye terminator in the form of fluorescent dye rhodamine (PRISM dyedoaxy reaction terminator cycle sequencing kit). After getting the results of sequencing, DNA sequences then aligned using NCBI Blast program.

Data analysis

Identification of nucleotide sequences carried by some analysis: Local alignment analysis of DNA sequencing results with existing data in GeneBank done with BLAST (Basic Local Alignment Search Tools) provided by NCBI (National Center for Biotechnology Information) through http://www.ncbi.nlm.nih.gov/blast. Furthermore, the nucleotide sequences of the DNA isolated was grouped using phylogenetic trees with Mafft Katoh program. Amino acid analyzed using Expasi program. Finally organisms that were analyzed can be determined its species.

RESULTS AND DISCUSSION

A. Isolation of genomic DNA CPBP (Conopomorpha camarilla Snell.)

Genomic DNA pest Cocoa Pod Borer Pests (CPBP) was isolated from individual intact with CTAB method. The result of DNA electrophoresis of CPBP sample shown in Figure 1. Based on these results indicate good quality DNA bands because DNA band shown quite clear and not show the banding pattern of DNA is degraded.

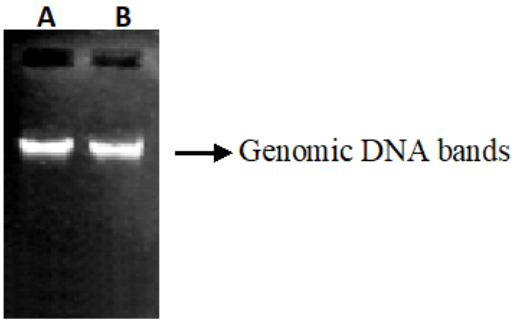

Figure 1: Results of electrophoresis of genomic DNA CPBP (*C. camarilla*). A = B = 100 ng

The concentration of genomic DNA isolated from the CPBP sample was 4,500 µg / ml with a purity level of 1.95. These results indicate that the degree of purity of genomic DNA is quite good so it can be directly used in the PCR amplification process. DNA purity level is said to be good if the value of the ratio of A_{260}/A_{280} ranged from 1.8 to 2.0. If the value of the ratio is below 1.8, then the DNA is contaminated with protein and should be re-extracted. Spectrophotometer measurement results are detailed in Table 1.

Table 1: Test results spectrophotometric

No	Sample	Absorbance		Purity of DNA	[DNA](µg/ml)
		λ260	λ280		
1	Blanko	0.000	0.000	-	-
2	CPBP	0.045	0.023	1.951	4.500

ISSN: 0975-8585

EF-1α gene amplification by PCR

Result of EF-1α gene amplification showed targets DNA bands with clear and thick that forms a single band with a length of about 600 bp products. This amplicon corresponds to the target amplification of EF-1α fragments produced by primer pbk-F and pbk-R primers.. Results visualization EF-1α gene amplification can be seen in Figure 2.

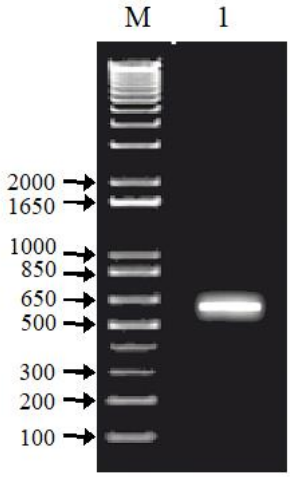

Figure 2: Results of electrophoresis of PCR products of DNA samples CPBP with *pbk*-F and *pbk*-R primers on a 1% agarose gel (1), 1 kb ladder (M).

DNA sequencing and Sequence Analysis

Sequencing is the stage of determining the nucleotide sequences in a DNA molecule. The number of nucleotides results of PCR using pbk-F and pbk-R primer is 601pb with a sequence as shown in Figure 3. The sequencing results were analyzed using BLASTN program on site www.ncbi.nih.gov to determine the level of similarity of PCR produk sequence with gene sequences in GeneBank.

```
  1 CTCTACTGAGCCCCCATACAGCGAAGCCAGATTCGAGGAAATCAAGAAGGAAGTA 55
 56 TCCTCATACATCAAGAAGATCGGCTACAACCCAGCTGCCGTCGCTTTCGTACCCA 110
111 TTTCTGGCTGGCACGGAGACAACATGCTGGAGCCCTCTACCAAAATGCCCTGGTT 165
166 CAAGGGATGGTCTGTTGAGCGTAAGGAAGGTAAGGCCGACGGCAAGTGCCTGATC 220
221 GAAGCTTTGGACGCCATCCTGCCCCCTGCCCGCCCCACAGACAAGGCTCTCCGTC 275
276 TTCCCCTCCAGGACGTCTACAAAATCGGTGGTATTGGAACGGTGCCCGTCGGCCG 330
331 TGTGGAGACTGGTGTCCTGAAGCCCGGTACCATTGTTGTATTCGCCCCCGCTAAC 385
386 ATCACCACTGAAGTCAAGTCCGTGGAGATGCACCACGAAGCTCTCCAAGAGGCTG 440
441 TACCCGGAGACAACGTTGGTTTCAACGTTAAGAACGTCTCAGTCAAGGAATTGCG 495
496 TCGTGGTTACGTCGCCGGTGACTCAAAGAACAACCCACCCAAGGGTGCCGCTGAC 550
551 TTCACTGCTCAGGTTATTGTCTTGAACCACCCTGGTCAAATCTCCAACGGT      601
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Figure 3: Nucleotide sequence of EF-1α gene fragment of CPBP sample.

Based on the Blast-n analysis on the sequence of EF-1α fragments from the CPBP sample compared with the gene sequence encoding EF-1α from Gene bank showed a 100% similarity. The similarity values of 99-100% are expressed as the same species, while similarity values of 89-99% belong to the same genus [13], and similarity values of 80-88% are still classified in the same family [14]. The percentage value of similarity is determined by the arrangement of nucleotide similarity between EF-1α fragments of CPBP and the existing data in the gene bank. The value of the similarity of the EF-1α gene sequences samples CPBP against EF-1α gene sequences in Gene bank can be seen in Table 2. Based on the percentage of similarity values, CPBP sample is the same species as C. camarilla because it has a value of 100% similarity with C. camarilla in GeneBank.

ISSN: 0975-8585

Table 2: Nucleotide and amino acid similarities EF-1α gene fragment of CPBP sample with some nucleotides and amino acids on genbank.

Organism	Gene Bank Acc. number	Nucleotide similarity (%)	Amino acid similarity (%)
CPBP sample	-		
Conopomorpha camarilla	EU727205	100.0	100.0
Conopomorpha camarilla	EU708727	100.0	100.0
Conopomorpha camarilla	EU644662	100.0	100.0
Gluphisia septentrionis	AF151603	92.0	97.5
Tuta absoluta	KJ746735	92.0	99.0
Keiferia lycopersicella	KJ746710	91.0	97.5
Symmetrischema capsicum	KJ746694	90.0	96.0

Mapping of Retrictive Enzyme Sites

Restriction enzyme site map of EF-1α gene fragment of CPBP sample processed using NEB cutter program can be seen in Figure 4.

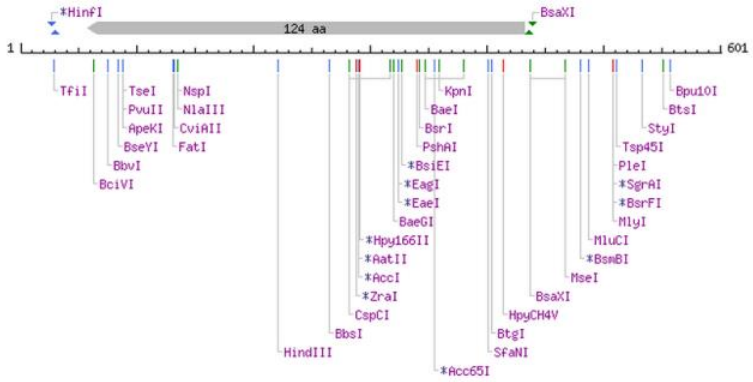

Figure 4: Restriction enzyme site map of EF-1α gene fragment of CPBP sample

The analysis showed that the sequence of EF-1α gene fragments from the CPBP sample had 42 restriction enzyme cutting sites. The classification of the enzyme is determined by the pattern of cutting through the site http://rebase.neb.com. The classification results show that enzymes are classified as restriction enzyme of endonuclease type II. These enzymes have three cutting patterns, namely blunt-ended DNA fragments, 5' sticky-end DNA fragments, and 3' sticky-end DNA fragments. The cutting pattern of a blunt-ended DNA fragment is owned by PvuII, ZraI, PshAI, HpyCH4V, and MlyI enzymes; the cutting pattern of 5' sticky-end DNA fragments is owned by the enzyme TfiI, BseYI, ApeKI, TseI, FatI, CviAII, HindIII, BbvI, EagI, AccI, Acc651, BtgI, SfaNI, BsmBI, MluCI, Tsp45I, MseI, EaeI, BsrFI, SgrAI, PleI, StyI, BbsI and Bpu10I; while the cutting pattern of 3' sticky-end DNA fragments is owned by BtsI, BsaXI, KpnI, BaeI, BsrI, BsiEI, BaeGI, CspCI, NspI, NlaIII, AatII, Hpy16611 and BciVI enzymes. These enzymes are one of the characters that can distinguish EF-1α gene fragments of CPBP sample from other species.

Restriction enzyme is an endonuclease that has the ability to cut DNA fragments specifically and purposefully. Restriction maps can be used for alternative approaches in phylogenetic analysis between species [15]. Restriction enzymes cut off the double strand DNA by breaking the covalent bond between the phosphates of a deoxyribonucleotide with the sugar from the internally binding deoxyribonucleotide (endonuclease) or degrading the DNA polynucleotide one by one from the end of the DNA molecule (exonuclease) [16].

ISSN: 0975-8585

Phylogenetic Analysis

The construction of phylogenetic tree in this article uses Neighbors-joining algorithm which is a consensus tree from bootstrapping 100 times replication of sequence data. Bootstrapping function to test the stability of clustering in phylogenetic tree construction; bootstrap can produce a number of random phylogenetic trees. The consensus program can then calculate how many times the sequences are clumped from random trees and this value can be used as a reference for the stability of phylogenetic trees.

The construction of phylogenetic trees based on DNA sequences indicates four groups with 100% confidence values. This value explains that the composition of the organism has been stabilized, meaning that even if 100 times the composition of the organism in the tree is turned back, the composition of the organism remains in its respective branches. According to [17] that a group with a bootstrap value of ≥ 95% in a branch can indicate the level of trust of a branch. The EF-1α gene fragment of CPBP sample is in the same group as C. camarilla. This suggests that the CPBP sample belongs to the same species as C. camarilla (Figure 5).

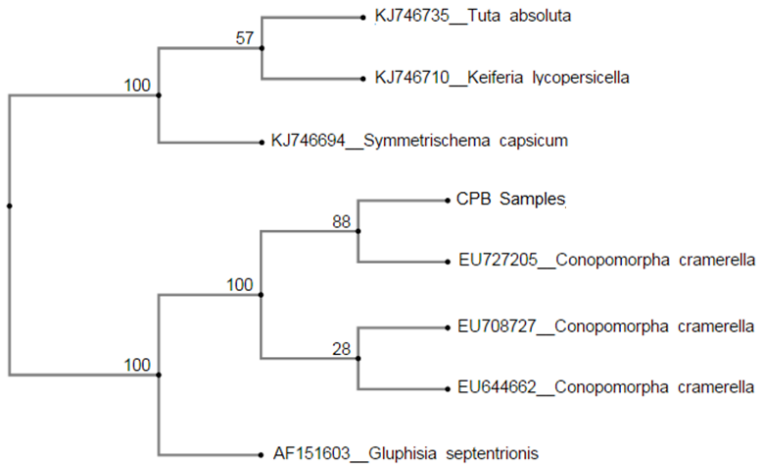

Figure 5: The phylogenic tree constructed by the Neighbors-joining algorithm shows the kinship relationship between nucleotide of EF-1α gene fragment of CPBP sample with some nucleotides in gene bank. Figures on branching indicate bootstrap values based on Neighbour-joining analysis with 100 replication times

Analysis of Amino Acids

The results of the amino acid deduction of EF-1α gene sequences from the CPBP sample using the expasy program resulted in 200 amino acids with the amino acid composition are 0.5% cysteine (C), 1.5% tryptophan (W), 5.5% leucine (L), 8.5% proline (P), 2% histidine (H), 2% glutamine (Q), 3.5% arginine (R), 6% isoleucine (I), 1.5% methionine (M), 4.5% threonine (T), 2.5% tyrosine (Y), 5% asparagin (N), 8.5% lysine (K), 5.5% serine (S), 10.5% valine (V), 8.5% alanine (A), 4% aspartic acid (D), 7.5% glutamic acid (E), 9.5% glycine (G), and 3% phenylalanine (F). The largest amino acid composition of the EF-1α gene sequence of CPBP samples is valine by 10.5%. The amino acid sequence is shown in Figure 6.

STEPPYSEARFEEIKKEVSSYIKKIGYNPAAVAFVPISGWHGDNMLEPSTKMPWFKGWSVERKEGKADGK
CLIEALDAILPPARPTDKALRLPLQDVYKIGGIGTVPVGRVETGVLKPGTIVVFAPANITTEVKSVEMHHEAL
QEAVPGDNVGFNVKNVSVKELRRGYVAGDSKNNPPKGAADFTAQVIVLNHPGQISNG

Figure 6: Amino acid deduction of EF-1α gene fragment of CPBP sample.

Based on the result of amino acid alignment of the EF-1α gene fragmen of CPBP sample with EF-1α gene of C. camarilla showed 100% similarity value, Tuta absoluta 99%, Gluphisia septentrionis 97.5%, Keiferia lycopersicella 97.5%, and Symmetrischema capsicum 96 % (Table 2).

Based on Multiple sequence alignment the amino acid sequence encoded by the EF-1α gene fragment

ISSN: 0975-8585

of CPBP sample is not different from the amino acid sequence encoded by the EF-1α gene fragment of C. camarilla in the gene bank, while compared with the amino acid sequence of Tuta absoluta, Gluphisia septentrionis, Keiferia lycopersicella and Symmetrischema capsicum showed a difference (Table 2 and Figure 7).

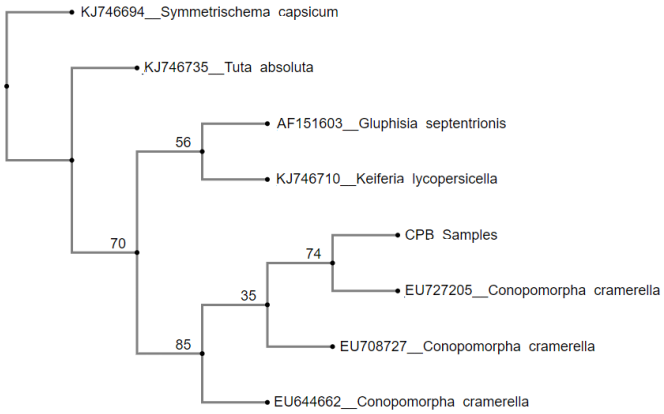

Figure 7: The phylogenic tree constructed by the Neighbor-join algorithm shows a kinship relationship between the nucleotide EF-1α gene fragments of the CPBP sample with some of the nucleotides in the gene bank. Figures on branching indicate bootstrap values based on Neighbor analysis-joined by 100 replication times

The hydrofobicity profile of Kyte and Doolittle gives an illustration that the hydrophobic area is above the 0 line while the hydrophilic area is below the line 0. Based on the hydrophobicity result indicates that the amino acid sequence of the EF-1α gene fragment of the CPBP is in the hydrophilic and hydrophobic areas, but the dominant is in the area hydrophilic (Figure 8).

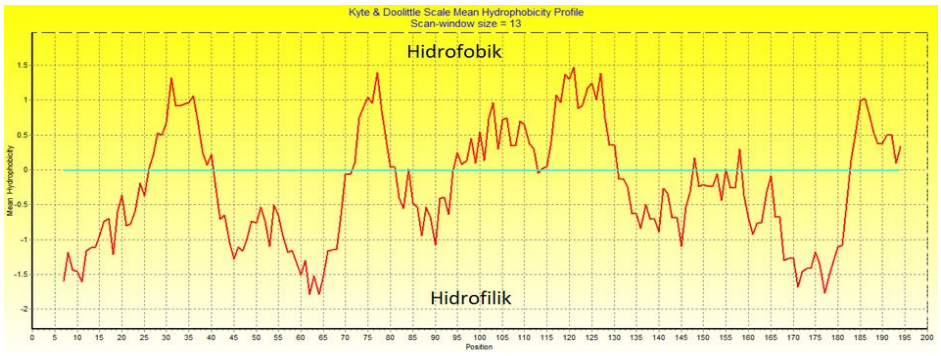

Figure 8: The hydrophobicity profile of the amino acid sequence of the EF-1α GHG gene fragment

ACKNOWLEDGMENTS

We would like to thank the Ministry of Research, Technology, and Higher Education who have provided financial support for this research in the form of a Competitive Grant entitled Morphology and Molecular Analysis of Cocoa Plants (Theobroma cacao L.) Southeast Sulawesi's Origin of Pest and Disease Resistance.

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