Jurnal Ilmiah Kesehatan Sandi Husada

Volume 13 Number 2 Desember 2024
DOI 10.35816/jiskh.v13i2.1205

ORIGINAL ARTICLES OPEN ACCESS

Molecular characterization of selected endophytic fungi isolate IDGG 3 leaf galing galing (cayratia trifolia L.) with the polymerase chain reaction method

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Received: 12 September 2024 ○ Revised: 04 November 2024 ○ Accepted: 01 December 2024

ABSTRACT

Background: Endophytic fungi live in plant tissues and are usually not harmful to their host plants. One of the host plants for endophytic fungi is bush grape leaves (Cayratia trifolia L.).

Objective: The research aimed to determine the molecular characteristics of the endophytic fungi isolates selected IDGG 3 of bush grape leaves.

Methods: The test used the polymerase chain reaction method to determine the species-level characteristics using internal transcribed spacers (ITS) 1 and 4. The DNA band was successfully amplified with 500 base pairs with 3000 markers.

Results: The fungi isolates selected IDGG 3 of bush grape leaves in molecular identification based on the Basic Local Alignment Search Tool (BLAST) analysis on Genebank NCBI that the IDGG 3 samples of bush grape leaves had a similarity level of 99%, namely Fusarium incarnatum JL5-2, Fusarium incarnatum JL3-4- 1, Fusarium incarnatum CBB-2, Fusarium incarnatum JL3-3, Fusarium incarnatum CBA-3, Fusarium incarnatum CBB-1, Fusarium incarnatum CBA-2, Fusarium chlamydospore, Fusarium cf. Incarnatum, and Fusarium sp.

Conclusion: The results of the molecular characteristics of the selected endophytic fungi isolate IDGG 3 galing-galing leaves (Cayratia trifoliata L.) have the closest degree of kinship with the species Fusarium incarnatum JL3-4-1. The importance of the PCR method in the molecular characterization of endophytic fungi, as well as opening opportunities for further exploration of the biotechnological potential of endophytic fungi from Cayratia trifolia L.

Keywords: bush grape leaves (cayratia trifolia L.), endophytic fungi, molecular identification, PCR.

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INTRODUCTION

Indonesia is one of the countries with the highest biodiversity in the world. It is estimated that there are around 30,000 types of plants, and 7000 of them have medicinal properties (Navia *et al.*, 2022). Each plant contains secondary metabolite compounds, which have not been widely used as a source of active ingredients, and the compounds contained in them are vast (Twaij and Hasan, 2022). Endophytic fungi are one of the main sources of secondary metabolites that have medicinal properties. The galing-galing plant (Cayratia trifolia L.) is from the Vitaceae family, commonly known as fox grape. This plant is usually found in lowlands in tropical and subtropical areas in Asia, India, and Australia. The whole plant can be used as a treatment for tumours, neuralgia, diuretics, and vaginal discharge. Leaves, roots, and seeds treat ulcers (Ashton and Zhu, 2020).

Most characters are complex to analyze because they do not have a simple genetic control system. Therefore, molecular analysis is needed. Molecular engineering provides an opportunity to develop and identify genetics. Molecular using DNA markers has succeeded in forming molecular markers capable of detecting specific genes and traits and evaluating diversity at the genetic level (Zhang *et al.*, 2022). Identification of endophytic mould using molecular identification has a high level of sensitivity and is fast and accurate. The identity of endophytic moulds can be known down to the species level based on DNA sequencing analysis (Mirsam *et al.*, 2022). Wheat microbiome harbours diverse microbial communities and plays a vital role in maintaining wheat physiology and offering protection from biotic and abiotic stresses (H. Ambo Lau and Herman, 2020). Several research findings indicated that the wheat microbiome encompasses predominantly fungi, bacteria, viruses, actinomycetes, cyanobacteria, protozoa, archaea, etc., which performed myriads of advantageous activities, including bio-management of crop pathogens, abiotic stress amelioration, as well as plant growth promotion under adverse conditions. This chapter attempts to provide comprehensive and up-to-date insights on wheat microbiome research, emphasizing emerging microbiome-based sustainable solutions for profitable and quality wheat production under every changing climate (Mahapatra *et al.*, 2020).

Endophytic fungi are microorganisms that live symbiotically within plant tissues without causing damage to their hosts (Myovela, Hussein and Tibuhwa, 2024). They play an essential role in helping plants cope with various unfavourable environmental conditions, including pathogen attacks, drought, and nutrient deficiencies. In addition, endophytic fungi are known to be able to produce a variety of beneficial bioactive compounds, such as antibiotics, anticancer, and antimicrobials, thus attracting attention in the fields of biotechnology and pharmaceutical research (Pokhriyal *et al.*, 2024). Cayratia trifolia L., known by its local name "galing-galing," is a vine often found in various tropical regions, including Indonesia. This plant can be a source of traditional medicine ingredients because of its secondary metabolite content, such as flavonoids and alkaloids.

Further research is needed to identify the microorganisms that live inside these plants, especially endophytic fungi, which may play a role in synthesizing bioactive metabolites (M.R. *et al.*, 2024). Characterization of endophytic fungi from the plant Cayratia trifolia L. is important to explore its therapeutic and ecological potential. One of the methods used to identify endophytic fungi accurately is Polymerase Chain Reaction (PCR). PCR is a molecular technique that allows for specific DNA amplification, so it is beneficial for identifying microorganisms based on their genetic profile (Varghese *et al.*, 2024).

Morphological characters have long been used in many phylogenetic studies. The development of techniques in molecular biology is getting more and more rapid, such as polymerase chain reaction and DNA sequencing (Ibrahim *et al.*, 2025). The use of DNA sequences in phylogenetic research has increased rapidly and has been carried out at all taxonomic levels, such as families, genera, and species (Antil *et al.*, 2023). Molecular phylogenetics combines molecular biology techniques with statistics to reconstruct phylogenetic relationships.

So, currently, there are many searches for new antibacterial compounds. In this case, plants are widely used as targets for new drug discovery (Wibberg *et al.*, 2021). The development of the use of traditional medicine, predominantly plants, can help improve the degree of public health. One of the plants that can be used as a source of endophytic fungi isolates is the leaves of galing-galing (Cayratia trifolia L.). This study aims to determine the molecular characteristics of endophytic fungal isolates selected by IDGG 3 from shrub grape leaves.

RESEARCH METHODOLOGY

Tools and Materials

The tools used are an autoclave (SMIC Model YX-280 B), Petri dishes (Normax), Erlenmeyer glasses (Iwaki Pyrex), incubators (mammerts®), sprites lamps, laminar Air Flow (Envirco®), refrigerators, micropipettes, ovens (Memert), Polyemerase Chain Reaction. The materials used are aquadest, 70% ethanol, IDGG 3 selected endophyte fungi isolate, agarose gel, Potato Dextrose Agar (PDA) medium, proteinase enzyme K, ZymocleanTM Gel DNA Recovery Kit, buffer, primer (Forward Internal Transcribed Spacer 1 and Reverse Internal Transcribed Spacer 4).

Sample preparation

Purification of endophytic fungi isolate

Purification is carried out by transferring each mushroom isolate to a new PDA medium. Then, it is incubated for 5-7 days at room temperature. Purification was carried out until a single pure fungal isolate was obtained, and macroscopic analysis was carried out to distinguish pure fungal isolate strains.

Macroscopic examination.

Macroscopic examinations include colony colour and surface (granular, such as flour, mountainous, slippery), texture, zoning, growing regions, radial and concentric lines, colony reverse colour, and exudate drops.

Molecular Characterization Testing

DNA Extraction

DNA extraction separates the DNA genome from other molecules in the cell. DNA extraction is carried out at this stage using the ZymocleanTM Gel DNA Recovery Kit, a tissue DNA extraction kit. The mycelia part of the selected endophytic fungi is cut into small pieces, weighed as much as 25 mg, and placed in a 1.5 mL effendorphic tube. Then, 180 uL ATL buffer is added, facilitating the lysis process. 20 UL Proteinase K was added and then crushed with micropastels. Proteinase K functions to break peptide bonds on proteins found in mycelium. Then, it is incubated at a temperature of 600 C for 30 minutes so that the mycelia of the fungus is destroyed.

The next stage is lysis; at this stage, GBT buffer is added to the sample, and then the sample is incubated for 10 minutes at a temperature of 600 C to help accelerate the occurrence of lysis in cells. Next, absolute ethanol is added to collect DNA; this stage is also called DNA binding because, at this stage, the DNA of the sample is collected in case a high ethanol concentration will not damage the DNA. However, the higher the ethanol concentration, the stronger the ethanol collects the DNA obtained and transfers it into the GD column. The matrix on the GD colom will bind to the DNA while the contaminant will be suspended.

At this stage, washing uses a wash buffer to remove contaminants while the DNA remains bound in the matrix. In the next stage, the DNA rehydration process is carried out, which aims to melt or release DNA because DNA products are in the form of sediment. Adding Rehydration is

done with a pre-heated Elution buffer solution that can dissolve DNA. After the rehydration process is complete, DNA products are produced.

DNA amplification with Polymerase Chain Reaction technique

DNA amplification takes place in the following stages. The initial stage is 95o C denaturation for 3 minutes, 98o C denaturation for 10 seconds, an annealing temperature of 52o C for 30 seconds, and an extension of 680 C for 45 seconds. In the final stage of the PCR process, Final Extension is carried out for 3 minutes at a temperature of 75oC with 37 cycles. In the PCR stage, Forward ITS1 and Reverse ITS4 primers are used, which will specifically amplify the following targets: Forward Primer: (5'-TCCGTAGGTGAACCTGCGG-3'), Reverse Primer: (3'-TCCTCCGCTTATTGATATGC-5') (Mia, Endang, Anto, pp. 34-40).

Electrophoresis test

1 g is weighed and dissolved in 100 mL of TAE Buffer into Erlenmeyer, heated in the microwave for 2 minutes to boil, then added eight µL of ethidium bromide. The gel liquid is poured into an agarose printing container until it solidifies. 5 µL of each amplified sample PCR product was inserted into the well on the submerged agarose in a tank containing TAE Buffer. Electrophoresis process.

Sequencing and species identification tests using BLAST

Furthermore, to determine the nucleotide base arrangement, the purification results are sent to the 1st Base sequencing service provider company through the Molecular Biology company PT. Genetics Science Indonesia was then sent to Singapore. The sequences were analyzed with the help of a sequence program performed on a Bioedit device. The analysis results were then compared with the genes in the database through the BLAST® (https://blast.ncbi.nlm.nih.gov/Blast.cgi) facility. The BLAST facility predicted the arrangement of gene donor amino acid residues on the BLAST® website. The analysis of the BLAST results provides information. It verifies organisms or bacteria that have similarities with the DNA sequence of the sample so that it can be used for bacterial identification. The information from the BLAST results is in the form of Query Coverage and Maximum identity. Query coverage is the percentage of nucleotide length aligned with the database contained in BLAST. Max identity is the highest value of the percentage of identity or match between the query sequence and the aligned database sequence.

Phylogenetic analysis test

The data was then processed for phylogenetic analysis using the Neighbor-Joining Tree method. The search was conducted using an NCBI device by Genetics Science Indonesia on a Windows 10 computer.

RESULT

Table 1. **Macroscopic observation results of selected endophytic fungi isolate IDGG 3 galing galing leaves (Cayratia trifolia L.)**

Isolate	IDGG 3
Colony Form	*Round with scalloped margin* (Round with toothed edges)
Edge Shape	*Thread-like* (Thread-shaped)
Elevation Shape	*Raised* (arise)
Colour	White
Surface	Cotton
Central point	There is
Clear Coating	-
Colony Size	80,5 mm
Dew Point	There is
Background	Layered

The results in Table 1 describe the macroscopic observation of isolated endophytic fungi selected IDGG 3 leaves of galing-galing (Cayratia trifoliata L.) As a result of macroscopic results, the endophytic fungi isolate obtained is seen from the shape of the colony Round with a scalloped margin, the shape of the Thread-like edge.

Table 2. Results of DNA concentration measurement test at wavelength 260/280

Sample name	Wavelength (A) 260/280	Concentration ng/µl
IDGG3	1,95	78,1

The results in Table 2 describe the selected endophytic fungi isolate IDGG 3 galing-galing leaves (Cayratia trifolia L.) that have been measured using a UV-Vis spectrophotometer at a wavelength (λ) of 260/280 nm.

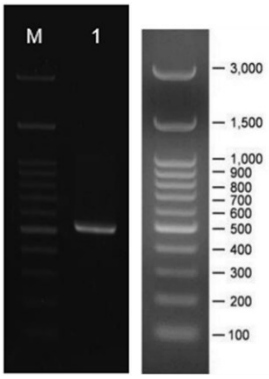

Figure 1. The results of the PCR product were visualized using electrophoresis of selected endophytic fungi isolate IDGG 3 leaves of galing-galing (Cayratia trifoliata L.). Description: (M) = Marker, (1) = sample

The results of electrophoresis testing show that the isolation of DNA molecules quite well characterizes the DNA molecules resulting from the extraction. The results of DNA amplification of the selected endophytic fungi isolate IDGG 3 leaves galing-galing (Cayratia trifoliata L.) produced a single band that was visible at a length of 500 base pairs with markers used 3000 base pairs from the results showed that the isolated selected endophytic fungi IDGG 3 leaves galing-galing (Cayratia trifoliata L.) was successfully amplified using Internal Transcribed Spacer Primers 1 and 4.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Fusarium incarnatum strain JL5-2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, a	958	958	99%	0.0	100.00%	MT563420.1
Fusarium incarnatum strain JL3-4-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene	958	958	99%	0.0	100.00%	MT563419.1
Fusarium incarnatum strain CBB-2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene,	958	958	99%	0.0	100.00%	MT563418.1
Fusarium incarnatum strain JL3-3 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, a	958	958	99%	0.0	100.00%	MT563417.1
Fusarium incarnatum strain CBA-3 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene,	958	958	99%	0.0	100.00%	MT563408.1
Fusarium incarnatum strain CBB-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene,	958	958	99%	0.0	100.00%	MT560226.1
Fusarium incarnatum strain CBA-2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene,	958	958	99%	0.0	100.00%	MT560219.1
Fusarium chlamydosporum isolate 22_2B10_7MN_547bp small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S	958	958	99%	0.0	100.00%	MK534502.1
Fusarium cf. incarnatum-equiseti strain PaB-3 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal	958	958	99%	0.0	100.00%	MN646258.1
Fusarium sp. MER-5 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal tr	958	958	99%	0.0	100.00%	MN864181.1

Figure 2. Identification results based on the level of DNA homology of selected endophytic fungi isolate IDGG 3 leaves of galing-galing (Cayratia trifoliata L.) with BLAST Analysis

The test results show that the image column includes the Description and the information title, which usually consists of genus, species, strain type, type of gene/DNA fragment, and type of DNA sequence completeness displayed per scattered information. Max Score is the total score obtained from the alignment between the input sequence/query and the database sequence. The value is obtained from the calculation of the matrix, which is a substitution matrix. The total score is obtained from all parallels between the input sequence/query and the parallel database sequence. Query Coverage is a percentage that describes how large/long the fit of the input sequence is when compared to the target DNA sequence.

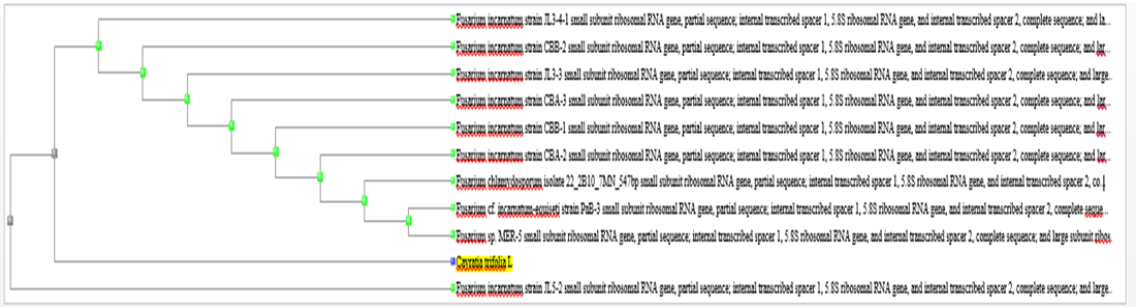

Figure 3. Phylogenetic Tree Results on DNA Isolate of Selected Endogenous Fungi Isolate IDGG 3 Leaves of Galing-Galing (Cayratia trifolia L.)

The results of phylogenetic tree testing using the Neighbor-Joining method showed that IDGG 3 isolate of galing-galing leaves (Cayratia trifolia L.) had a high similarity value and a closer kinship rate with Fusarium incarnatum JL3-4-1.

DISCUSSION

The researchers found that the molecular characteristics of the selected endophytic fungi isolate IDGG 3 leaf galing-galing (Cayratia trifoliata L.) had the closest degree of kinship with the species Fusarium incarnatum JL3-4-1. Based on research that has been carried out using DNA samples of selected endophytic fungi isolate IDGG 3 leaves galing-galing (Cayratia trifoliata L.), the success of amplification must have good quality, namely not smeared, not cut. In the research that has been carried out using the Internal Transcribed Spacer 1 and 4 primers, it is proven that the primer can amplify the selected endophytic fungi isolate mould IDGG 3 galing-galing leaves (Cayratia trifoliata L.) which is shown where the amplification results of the Internal Transcribed Spacer 1 and 4 primers in this study show the appropriate size, namely 500 base pairs So that it continues in the squeezing process. IDGG 3 endophytic fungi isolate taken from the leaves of Cayratia trifolia L. have been molecularly characterized using the Polymerase Chain Reaction (PCR) method with primers specific to ribosome genes (such as ITS). The DNA sequencing results obtained from the isolates are then analyzed using sequence databases, such as GenBank, through the BLAST (Basic Local Alignment Search Tool) tool. From the results of DNA sequence matching, the IDGG 3 fungi isolate has the closest degree of kinship with Fusarium incarnatum strain JL3-4-1 (Doyle *et al.*, 2020).

Fusarium incarnatum is a fungal species often found in various plants as a pathogen, endophyte, or saprophytic (Santos *et al.*, 2020). The existence of this fungus as an endophyte in Cayratia trifolia L. can provide different potentials, both in terms of symbiosis mutualism and the ability to produce beneficial bioactive metabolites. Fusarium incarnatum is also known for its ability to create a variety of bioactive compounds, including antimicrobial and anticancer compounds, which makes it essential in the context of biotechnology. The closest degree of kinship with Fusarium incarnatum JL3-4-1 suggests that IDGG 3 isolate may have genetic and phenotypic characteristics similar to this species. This can be interpreted from the results of ITS region sequencing, which is commonly used in phylogenetic fungi studies. The percentage of sequence similarity between IDGG 3 isolates and Fusarium incarnatum suggests that they belong

to a single phylogenetic cluster, which supports a close evolutionary relationship (Balamurugan *et al.*, 2024).

Because Fusarium incarnatum is known to produce bioactive compounds such as enzymes, antibiotics, and other secondary metabolites, IDGG 3 isolate may have similar capabilities (Stępień *et al.*, 2020). This opens up further research opportunities to explore the potential metabolites produced by these fungi, especially in medical applications, such as antimicrobials or other therapeutic agents. More in-depth research is needed to explore the specific metabolites produced by IDGG 3. Query Coverage is a percentage that describes how large/long the fit of the input sequence is when compared to the target DNA sequence (Xie *et al.*, 2022). If the DNA sequence is inserted through/covering the entire target DNA sequence in the NCBI database, then the percentage is 100%. E value is an expectation value, a measure of this alignment by chance. A good E value is close to zero. So, there is no possibility that a parallel incident occurred (Chatain, Boltenhagen, and Carmona, 2021).

The amplified products in the PCR are then sequenced to determine the nucleotide base sequence. Identification is carried out based on sequence data. The sequencing results obtained are DNA base sequences (Chang *et al.*, 2024). The alignment process uses the Basic Local Alignment Search Tool (BLAST) on the NCBI website. BLAST is a program that finds and analyzes the level of homology of organism sequences. Based on the results of the homology search for the ITS rDNA area. The results of the BLAST Homology search obtained information on the closest relatives in the isolate. Phylogenetic trees describe the kinship relationships between species in isolates to facilitate the analysis of identification results where phylogenetic trees are formed using BLAST result data by NCBI using the Neighbor-Joining method. The Neighbor-Joining method has the advantage that it is considered faster than other methods and more practical for analyzing large data sets (Aumüller, Bernhardsson and Faithfull, 2020).

Ecological Implications and Applications of Biotechnology

Endorphic fungi often play an essential role in increasing plant resistance to pathogens or stressful environmental conditions. The presence of Fusarium incarnatum as an endophyte in Cayratia trifolia L. may indicate a potential role in plant ecology, such as increasing disease resistance or improving nutrient absorption. In biotechnology applications, these isolates can be a possible source for developing new bioactive agents used in agriculture or pharmaceuticals. As a follow-up, further research is needed to understand the interaction mechanism between the endophytic fungi IDGG 3 and its host plant, Cayratia trifolia L., and further explore the bioactive metabolites that can be produced. Further characterization through other techniques, such as chromatography to study secondary metabolite profiles, is also critical to expanding knowledge regarding the potential applications of these fungi. (Zhang *et al.*, 2022)

CONCLUSION

Based on the results of the research that has been carried out, it can be concluded that the results of the molecular characteristics of the selected endophytic fungi isolate IDGG 3 galing-galing leaves (Cayratia trifolia L.) have the closest degree of kinship with the species Fusarium incarnatum JL3-4-1. The importance of the PCR method in the molecular characterization of endophytic fungi, as well as opening up opportunities for further exploration of the biotechnological potential of endophytic fungi from Cayratia trifolia L.

Conflict of Interest

No conflict of interest

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How to Cite: Wirada, N., Fitriana, F. and Rusli, R. (2024) "Molecular characterization of selected endophytic fungi isolate IDGG 3 leaf galing galing (cayratia trifolia L.) with the polymerase chain reaction method", Jurnal Ilmiah Kesehatan Sandi Husada, 13(2), pp. 297-305. doi: 10.35816/jiskh.v13i2.1205.