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Molecular Identification of Fungi carried by Chili Seeds (*Capsicum annuum* L.) from Tanjung Morawa B Village, North Sumatra

Dewi Novina Sukapiring(*)[1], Lily Novianty[1], Siti Nurlani Harahap[2]

[1] Department of Agricultural Cultivation, Faculty of Agriculture,
Universitas Nahdlatul Ulama Sumatera Utara;
[2]Department of Nutrition Science, Faculty of Health Sciences,
Universitas Nahdlatul Ulama Sumatera Utara;
Jl. Gaperta Ujung No.2, Tj. Gusta, Medan Helvetia, Kota Medan, Sumatera Utara, Indonesia Postcode 20125

***Corresponding Author**: dewi.novina88@gmail.com

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Abstract

Seed-borne fungi are fungi that are associated with seeds and can be a source of plant disease in the field. Therefore, this study aims to utilize DNA barcoding to identify seed-borne fungi isolates from Tanjung Morawa B village. This investigation took place at Tangerang's genetic science laboratory in Tangerang was the site of this investigation. Sequence and phylogenic tree building, PCR amplification with MyTaq HS Red Mix, 2X (Bioline, BIO-25048), and genomic DNA extraction with the Quick-DNA Magbead Plus Kit (Zymo Research, D4082) were the research methods used. The electrophoresis results showed that the DNA made from PCR using Primers ITS-1 and ITS-4 was of high quality. This was clear from the electrophorograms for both the TMIE isolate sample and the control. Using ITS-1 and ITS-4 for DNA amplification produced favorable results, with virtually no smears detected. The Basic Local Alignment and Search Tool (BLAST) program, which is connected to the Genebank database, was employed to analyze the sequence results in the form of a base sequence with a length of 581 base pairs. Based on the genetic similarity results of the isolate's DNA with Genebank, we determined that the TMIE isolate was 100% similar to Curvularia eragrostidis.

Keywords: *Curvularia eragrostidis; Deliserdang; DNA barcoding; Seed-borne fungi*

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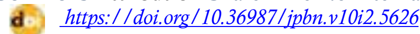

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INTRODUCTION

Seeds are a critical component of plant cultivation, as they can serve as a source of disease in areas where plants are cultivated. Seeds that are free of fungi, bacteria, viruses, and plant pests that can cause plant diseases are considered to be of high quality (ISTA, 2023). Numerous studies have previously reported the discovery of

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disease-causing fungi in association with seeds. *Aspergillus* sp., *Verticillium* sp., *Curvularia* sp., and *Penicillium* sp. are seed-borne fungi that infected chili seeds from Tamora B village (Sukapiring et al., 2023). *Alternaria* sp., *Aspergillus* niger, *Penicillium* sp., and four unidentified isolates were present in Mekongga rice seeds (Waliha et al., 2022). Seed-borne fungi, which infect seeds, have the ability to spread disease from seeds to plants in the field. Seed-borne fungi can be a source of disease spread in the field, reducing crop yields and spreading the disease to an area (ISTA, 2023). To mitigate disease transmission, it is critical to monitor the health of seeds (Aslam et al., 2017). To determine if the seeds are free from seed-borne fungi, it is necessary to isolate and identify the fungi on the seeds.

The morphological identification of chili seed-borne fungi from Tanjung Morawa B Village had previously identified the fungus groups *Curvularia* sp., *Verticillium* sp., *Aspergillus* sp., and *Penicillium* sp. The *Curvularia* sp. group had the highest infection rate (3%) (Sukapiring et al., 2023). Further research, specifically molecular identification of fungi carried by chili seeds from Tanjung Morawa B Village, is required in light of the findings of this study. We are currently in dire need of molecular identification methods that utilize RNA-seq-based tracing, DNA barcoding, and PCR to more accurately identify the organisms that cause plant diseases (Aslam, et al., 2017). Presently, molecular identification is prevalent, as evidenced by the widespread use of the DNA barcoding method to identify fungi, such as sponge-associated molds (Larasati et al., 2021). Molecular identification was also carried out on isolates of entomopathogenic fungi from Cianjur (Sari & Rosmeita, 2019). DNA barcoding was used to identify *Dothistroma septosporum* on *Pinus contorta* var. *latifolia*, P. *bankiana*, and their hybrids in northern Alberta, Canada (Feau, et al., 2021). Molecular identification of *Trichoderma* spp. from several rice varieties (Elita et al., 2022).

Morphological identification was carried out to observe colony colour, conidia shape, growth pattern and reproduction microscopically (Dharmaputra et al., 2018). Molecular identification is used to determine the exact identity of the fungus (Ulfa, et al., 2019). Currently, there is a significant need for molecular identification of fungi, particularly those carried by seeds, beyond mere morphological recognition. This is because it is necessary to know for certain the species of seed-borne fungi that infect seeds in a given area. This can provide new information about the presence of a fungus species in a particular area. Molecular identification is one way to determine the species of a fungus and its taxon of kinship, which is done by looking at the sequence of the desired fungal DNA nitrogenous bases (Novaldi, et al., 2018). Given this background, it is necessary to conduct research on the molecular identification of chili seed-borne fungi (*Capsicum annuum* L.) from Tanjung Morawa B Village, North Sumatra. The aim of this research was to identify isolates of seed-borne fungi obtained from Tanjung Morawa B village using DNA barcoding.

METHOD

The Genetic Science Laboratory in Tangerang, Banten, Indonesia carried out this research for a month before obtaining the results.

Propagation of Fungus Isolates

This study selected seed-borne fungi with the highest infection rates, previously identified through morphological analysis. The TMIE isolate from Tamora B village was the isolate that was molecularly identified due to its highest infection rate. Isolates were propagated in petri dishes containing PDA media and incubated at room temperature for ±7 days until fungal conidia formed, and the isolates were prepared to be sent to PT Genetic Science.

DNA Extraction

The DNA extraction process for TMIE isolates followed the instructions for the Quick-DNA Magbead Plus Kit (Zymo Research, D4082). A total of ± 50 mg of fungus isolate and 750 µl of DNA/RNA Shield were put into a lysis tube and homogenized by centrifuging at a speed of 10,000 x g for 1 minute. The centrifugation results were transferred to a new tube. Each sample was re-added back to Quick-DNA Magbinding Buffer and mixed on a shaker for 10 minutes. Remove the sample from the solution, transfer it, and collect the supernatant. Then washing was carried out twice using prewash buffer. The DNA was centrifuged at 10,000 x g for 1 minute. Move and incubate at 55 °C for 10 minutes. Next, add DNA Elution Buffer to each sample and centrifuge at room temperature for 5 minutes. The process moved the sample from the solution into a fresh tube, preparing the DNA template for the subsequent step.

PCR (Polymerization Chain Reaction)

The DNA amplification process utilized PCR MyTaq HS Red Mix, 2x (Bioline, BIO 25048), along with ITS-1 and ITS-4 primers. 50 µL of template DNA was mixed with 25 µL of MyTaq HS Red Mix solution, 2x ITS-1 and ITS-4 primers, 1 µL each, and water (dH2O) up to 50 µL. The PCR stage was carried out by predenaturation at a temperature of 95 °C for 1 minute in 1 cycle, then 25–35 cycles of amplification, denaturation at a temperature of 95 °C for 15 seconds, annealing for 15 seconds, and extension at a temperature of 72 °C. Next, electrophoresis was carried out for ± 90 minutes at a voltage of 75 volts. The results obtained were visualized with a UV Transilluminator and photographed with a UV digital camera.

Sequence and Phylogenic Tree Construction

Sequencing was carried out using Bi-directional sequencing by sequencing the DNA chain of the sample resulting from the previous PCR and comparing it with the DNA chain sequence in the Genebank database on the NCBI (National Center for Biotechnology Information) website. Making a phylogenic tree was also carried out using neighbor joining (an unrooted tree) in the NCBI Blast Tree.

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RESULTS AND DISCUSSION

DNA Amplification and Sequencing Results

Based on the electrophoresis results, the DNA produced from the PCR stage using Primers ITS-1 and ITS-4 is of good quality because the bands produced in the electrophorogram are visible for the TMIE isolate sample. Many reports suggest that ITS gene primers amplify DNA sufficiently to enable easy species differentiation. The amplicon results, which looked thick and clear, demonstrated that all fungal isolates could amp up the ITS region of their DNA well (Prihatini et al., 2019). Figure 1 displays the results of DNA amplification of TMIE isolates using ITS1 and ITS4, enabling them to proceed with the sequencing process. Table 1 displays the results of DNA tracing of TMIE isolates.

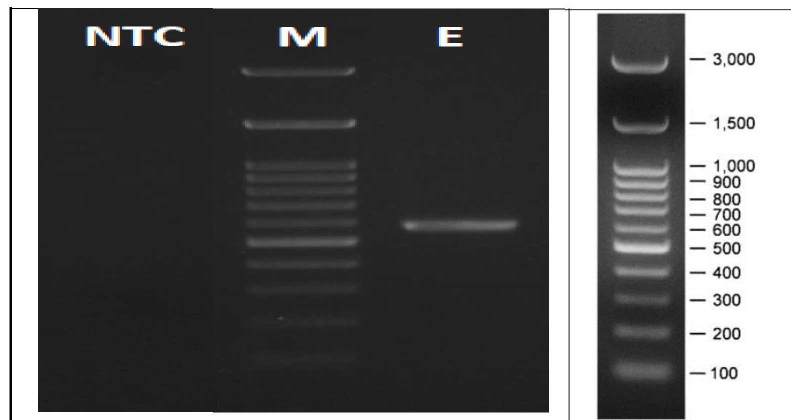

Figure 1. Results of DNA Amplification of TMIE isolates using primers ITS1 and ITS4

The results of DNA amplification of TMIE isolates using primers ITS1 and ITS4 showed clear DNA bands with a size of 600 bp. Amplification of fungal DNA that sticks perfectly to the DNA templates indicated by the results of DNA bands that look thick and not shaded (Jati et al., 2023). Using primers ITS1 and ITS4 on fungal isolates produced amplicons with a size range of 500–600 bp (Sari & Rosmeita, 2019). The initial stages in molecular identification are the stages of isolation of fungal DNA and PCR amplification of the ITS rDNA regn (Novaldi et al., 2018).

The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/) used the Basic Local Alignment and Search Tool (BLAST) program to analyze the base sequence results. The National Center for Biotechnology Information (NCBI) collects DNA data from a variety of plants, fungi, and animals worldwide, subsequently using Blast to search for sequences that are identical or closely similar to the plant species under study. The BLAST program can display the degree of homology between the sample base sequence and the base sequence input into Genebank, identifying species with a 100% homology level as identical or closely similar. This can be attributed to the flawless execution of the DNA amplification process during the PCR process. Table 2 provides detailed information on accession numbers from the Genebank database on the NCBI (National Center for Biotechnology Information) website.

Table 1. DNA Sequencing Results of TMIE Isolates

No.	DNA sequencing
1	TCCGTAGGGG AACCTGCGGA GGGATCATTA CACAAAAAAT ATGAAGGCTG CAACCGCCAG
61	TTTTGGCGGG GAAGCTGAAT TATTTTTCAC CCATGTCTTT TGCGCACTTG TTGTTTCCTG
121	GGCGGGTTCG CCCGCCACCA GGACCACACC ATAAACCTTT TTTATGCAGT TGCAATCAGC
181	GTCAGTATAA CAAATGTAAA TCATTTACAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
241	TCGATGAAGA ACGCAGCGAA ATGCGATACG TAGTGTGAAT TGCAGAATTC AGTGAATCAT
301	CGAATCTTTG AACGCACATT GCGCCCTTTG GTATTCCAAA GGGCATGCCT GTTCGAGCGT
361	CATTTGTACC CTCAAGCTTT GCTTGGTGTT GGGCGTTTTG TCTTTGGCTT TTGCCCCAAA
421	GACTCGCCTT AAAACGATTG GCAGCCGGCC TACTGGTTTC GGAGCGCAGC ACATTTTTGC
481	GCTTGCAACC AGCTAAAGAG GCCAGCAATC CATCAAGACC TTCTTCTCAC TTTTGACCTC
541	GGATCAGGTA GGGATACCCG CTGAACTTAA GCATATCAAT A

Table 2. BLAST Sequence Results of TIME Isolate Fragments

No	Description	Max Score	Total Score	Query cover	E Value	Per. Ident	Accession
1	*Curvularia eragrostidis* isolate CATAS-CE02	1068	1068	100%	0.0	99.83%	MT079201.1
2	*Curvularia eragrostidis* isolate CATAS-CE01	1068	1068	100%	0.0	99.83%	MT071747.1
3	*Curvularia sacchari-officinarum* strain TFL-8.1	1068	1068	100%	0.0	99.83%	OR061067.1
4	*Curvularia bannonii* strain CN021G8	1068	1068	100%	0.0	99.83%	ON074888.1
5	*Curvularia eragrostidis* strain 846	1066	1066	99%	0.0	100.00 %	KT933668.1
6	*Curvularia sacchari-officinarum* strain TFL-5.1	1064	1064	100%	0.0	99.66%	OR061066.1
7	*Curvularia eragrostidis* isolate DFF	1062	1062	99%	0.0	99.83%	MN890020.1
8	*Curvularia bannonii* strain CN024C4	1062	1062	100%	0.0	99.66%	ON074977.1

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No	Description	Max Score	Total Score	Query cover	E Value	Per. Ident	Accession
9	*Curvularia eragrostidis* strain 1167	1061	1061	99%	0.0	99.83%	KT933675.1
10	*Curvularia eragrostidis* strain 1172	1061	1061	99%	0.0	99.83%	KT933667.1
https://www.ncbi.nlm.nih.gov/nuccore/MT079201.1,MT071747.1,OR061067.1,ON074888.1,KT933668.1,OR061066.1,MN890020.1,ON074977.1,KT933675.1,KT933667.1							

Percentage identity is a percentage that shows how suitable the input DNA sequence is with the target DNA sequence. Based on Table 2, it can be seen that the percentage of query cover of TMIE isolate with 99% homology level and percentage identity of TMIE isolate has 100% similarity with *C. eragrostidis* strain 846 with accession KT933668.1. Similar research also explains that the query cover parameter of GMP 2 isolate has similarities with Bacillus cereus strain ATCC 14579 with a homology level of 98% which indicates the total length of the nucleotide sequence of GMP2 isolate is good enough to be aligned with the nucleotide sequence database in the NCBI gene bank (Nontji et al., 2022).

Table 2 also indicates that the E-value of the TMIE isolate is 0 (zero), which demonstrates that the match is significant. A low e-value can mean that the sequences studied have a high level of sequence homology and conversely a high e-value indicates a low level of homology (Sandy et al., 2015). Figure 2 displays the results of using a phylogenetic tree to compare the relationships of TMIE isolates.

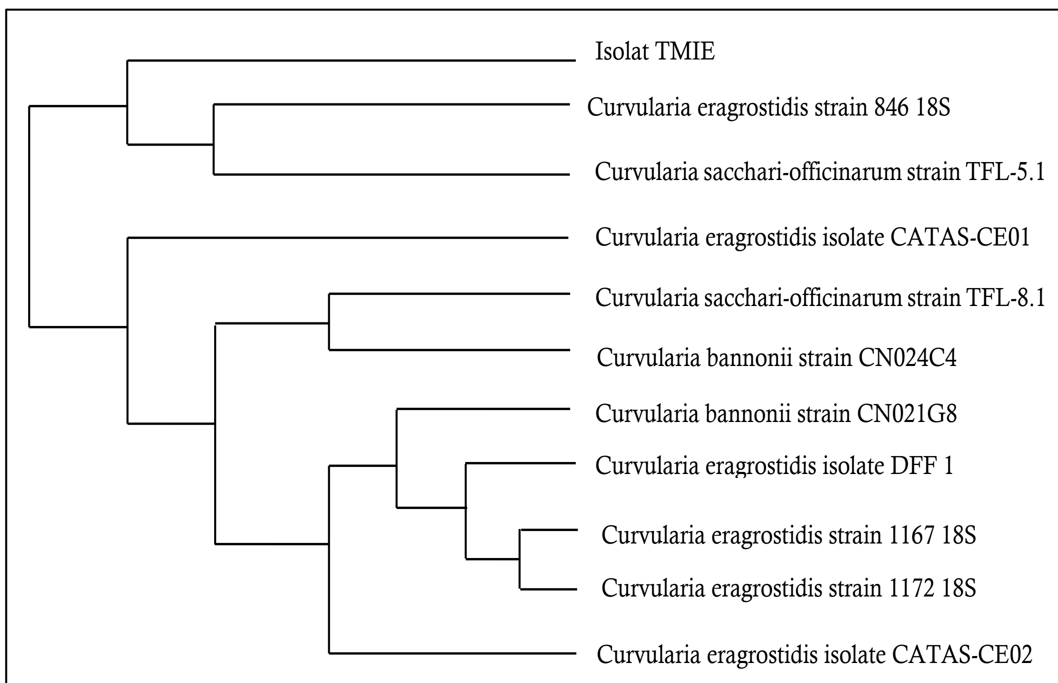

Figure 2. Phylogenetic tree based on DNA Barcoding with the ITS locus of TMIE Fungal Isolate samples.

In constructing phylogenetics, outgroup species obtained from genebanks with taxa that are not too far or close are needed. Outgroup species function as a comparison in determining which species are in the ingroup so that the phylogenetics formed are clear and robust to classify a kinship between individuals and species. Figure 2 shows that the TMIE isolate is related to *C. eragrostidis* strain 846 with a similarity percentage of 100%. The fungal isolates found were concluded to be very similar to the fungal data in Genbank because the percentage of similarity was around 97%-100%. (Alwakeel, 2017). The morphological identification results of TMIE isolates that have been published previously are *Curvularia* sp. Sukapiring et al., (2023), based on this, molecular identification was carried out in this study and the results obtained were the same as previous studies, that the TMIE isolate was a *Curvularia* group with the species *C. eragrostidis*.

Molecular identification research of seed-borne fungi is essential to determine the species of a fungus so that appropriate control of disease spread can be carried out. Identification based on morphological and biological characters can lead to incorrect species determination and take a long time, so it is crucial to do molecular detection of seed-borne fungi (An et al., 2023). Recommended for identification of fungi where possible by morphological and molecular identification (Raja et al., 2017).

It has now been reported that *C. eragrostidis* is the cause of leaf spot disease in oil palm seedlings and plants (Azis & Utoya 2014; Febriani & Kasiamdari, 2023). No journals have been found that report *C. erasgrostidis* as a species of chilli seed-borne pathogenic fungus yet, but *Curvularia* sp. group has been widely reported as a chilli seed-borne pathogenic fungus (Sukapiring, 2024), Soybean (Ramdan & Kalsum, 2017), Paddy (Zahara & Pamekas, 2022; Mulyani et al., 2023), Beans (Gupta et al., 2017), and was also found as a pathogen on the fruit of red chilli plant (Wakhidah et al., 2021). The results of this study provide new information that *C. eragrostidis* is a chilli seed-borne fungus found was located in the village of Tanjung Morawa B North Sumatra, and can be the initial information related to control techniques for the spread of plant disease *C. eragrostidis* in the field in the future.

CONCLUSION

The research concludes that the ITS amplifies the DNA resulting from PCR, enabling it to proceed to the next stage. In this study, the ITS barcode gene locus could be an excellent barcode candidate for differentiating types. The ITS1 and ITS4 barcode applications are able to distinguish types of isolates, namely at the species and genus levels. The phylogenic construction sorts isolates into groups based on their relatedness to each other. It shows that the TMIE isolate species, *C.eragrostidis*, has a similarity score of 100%. Therefore, the results of this research will bridge the gaps in the NCBI sequence list and serve as a foundational database for conservation efforts. Further research needs to be conducted to determine whether *C. eragrostidis* can cause disease.

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