Molecular Reidentification of Endophytic Fungus LBKURCC40 Isolated from Dahlia's Tuber (*Dahlia Variabilis*) Using the Sequence of ITS rDNA

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Molecular Reidentification of Endophytic Fungus LBKURCC40 Isolated from Dahlia's Tuber (*Dahlia Variabilis*) Using the Sequence of ITS rDNA

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Abstract: Endophytic fungus LBKURCC40 isolated from dahlia tubers (*Dahlia variabilis*) was morphologically and molecularly identified. Molecular identification was carried out using ITS4 and ITS5 primers, furthermore, the homology level of LBKURCC40 isolates was checked using BLAST program and the suitability of BLAST results was tested with the TrichOKEY program. The results of showed that LBKURCC40 isolates were identified as *Trichoderma asperellum* with a homology level of 99%. Phylogenetic analysis was carried out with N-J tree bootstrap test with 10,000 repetitions, and LBKURCC40 isolate was obtained to have the closest kinship to *Trichoderma asperellum* strain Tr266B.

Keywords: dahlia, endophytic fungus, ITS, Trichoderma

1. Introduction

Dahlias are tubular horticultural plants that contain compounds with high bioactivity. Endophytic microbes in dahlia plants are known to produce compounds with the same bioactivity as dahlia plants. Continuous interaction of dahlia tuber with microbes such as symbiotic mutual fungi produces endophytic fungi with the same ability as the host. The ability of these endophytic fungi provides an alternative to overcome drug resistance, and obtain the most desirable plant properties [11, 12, 14].

Endophytic fungi are known to correlate with their hosts in the ability to produce secondary metabolites. The type and amount of secondary metabolites produced by endophytic fungi is influenced by ecological or environmental conditions, such as temperature, humidity, and soil nutrients [8]. Some compounds produced by endophytic fungi have biological activities, such as alkaloids, terpenoids, and phenolics [16]. In agriculture, endophytic fungi can be used as biocontrol agents for various pathogenic microbes, insects, nematodes and pests [17]. In industry, endophytic fungi can be used to produce enzymes such as amylase, lipase, protease, and inulinase [2, 15].

In this study the identification of endophytic fungus in orange-flowered dahlia was carried out morphologically and molecularly. Morphological identification is done macroscopically and microscopically. Morphological identification can only identify to the genus level, and is not a reliable indicator for genetic identification between species, and is not sufficient to accurately identify species [7]. For that use advanced identification using molecular identification at the ITS region using primers ITS4 (forward) and ITS5 (revers). The ITS area is one of the fastest evolving regions and varies between species in the genus [6]. This area has several features so that it is easy to identify fungal molecules,

namely: (i) the length of the ITS area between 600 and 800 bp and can be easily strengthened using universal primers specific to the rRNA gene; (ii) has multicopy properties from rDNA repetition so that the ITS area is relatively easy to strengthen small, dilute or highly degraded DNA samples, and (iii) the ITS area varies greatly between fungal species with different taxonomies or even isolates from the same species [9].

The identification of endophytic fungus LBKURCC40 from orange flowering dahlia tubers has previously been carried out by previous researchers, but further identification is needed to ascertain the species of these isolates. Previous identification morphologically by Lorenita (2013) identified LBKURCC40 was *Monilia* sp. Morphological identification can only identify to the genus level, and has a low level of accuracy. This is because some species have almost the same morphological characteristics, so it's difficult to distinguish. To ensure the species from the isolates obtained, then the reidentification of LBKURCC40 isolates was morphologically and molecularly.

2. Methods

2.1. Morphology

Culture was grown on Potato Dextrose Agar (PDA) media at room temperature for 7 days. Morphological identification is done macroscopically and microscopically. Macroscopic morphology observation is the characterization of colonies and colony colors, while the microscopic identification of morphology is performed on conidia, hyphae, conidial heads, conidiophores and spores.

2.2. DNA extraction

LBKURCC40 isolate's DNA used is a collection of Enzyme, Fermentation, and Molecular Research Laboratory Universitas Riau which has been isolated Lorenita, *et. al* (2013) isolated from orange flowering dahlia tubers. Isolates were grown on Potato Dextrose Agar (PDA) media on petri dishes using the spread plate method. Micellia of fungi are isolated using the Wizard Genomic DNA Purification Kit (Promega). The results of DNA extraction continued to the electrophoresis stage using 0.8% agarose gel.

2.3. PCR amplification and DNA sequencing

The DNA obtained was used as a template for PCR amplification. PCR amplification using the ITS4 universal primer (5'-TCC GCT TAT TCC TGA TAT GC-3 ') as forward and ITS5 (5'-AGT AAA AGT ACA AGG-3' CGT) as revers. PCR amplification was carried out with a total volume of 50 µL containing 2 µL of DNA isolate, 10 µL of each primer concentrating 2 olmol, 5 µL dNTP concentrating 2mM, 10 µL buffer + 5 µL MgCl2, 7.75 µL H2O and 0.25 µL Go Taq DNA Polymerase. The reaction begins with a hot star at 95^{o}C for 5 minutes, denaturation at 94^{o}C for 1.5 minutes, annealing process at a temperature of 53^{o}C for 1 minute, and an extension process at 72^{o}C for 3 minutes. This PCR reaction process lasts for 35 cycles.

Sequencing results were verified with each other with the help of the BioEdit Sequence Alignment Editor and Analysis program for Windows 95/98/NT (Hall, 1999) and the proximity between each sequence was performed using clustal X version 2.0.9 (http://www.clustal.org) (Thompson, *et. al*., 1997). Phylogenetic analysis was carried out using the Neighbor-joining method using MEGA v software. 6. The confidence value of each branch is determined by 10,000 times the bootstap analysis.

3. Results and discussion

LBKURCC40 isolate was identified morphologically by looking at the growth and fungi colonies on PDA media. Morphological identification is done macroscopically and microscopically. Macroscopic morphological observations were performed by observing colony color and colony growth, while microscopically observing hyphae whether septic or not, transparent or cloudy, colored or not.

Morphological identification results showed that the LBKURCC40 isolate was *Tricoderma* sp, with macroscopic as follows, at the beginning of the incubation the isolate would be white, and then it would turn into dark green, with rapid growth. Whereas the results of identification microscopically indicate characteristics of mycelium having septa, branching conidiophores, and conidia in round shape. This characteristic is in accordance with that reported by Saryono, *et. al* (2018). Saryono, *et. al* (2018) reported *Trichoderma* had conidiophore branching down the recurrent lateral branch, conidia round to

oval-shaped spring, early colonies white and greenish, and dark yellow or dark green mycelium color. The results of the identification of the morphology of isolates LBKURCC40 can be seen in **Figure 1.**

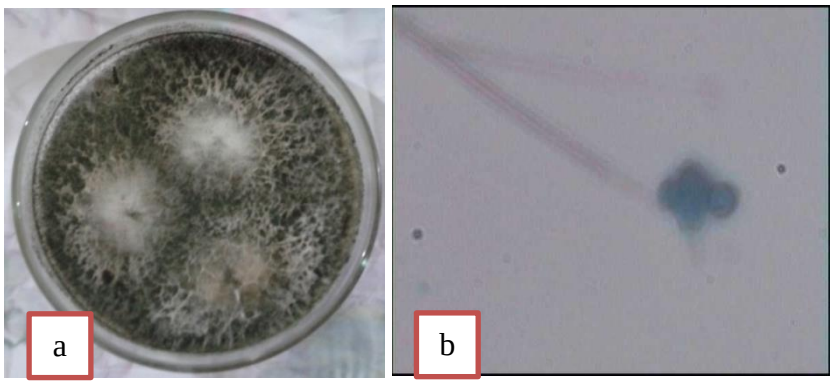


Figure 1. Morphological characteristics of LBKURCC40 fungi (a) Macroscopic (b) Microscopic with 40x magnification

Figure 1. Macroscopic and microscopic identification of morphological and LBKURCC40 isolates was *Trichoderma* sp. The results of this identification are different from the identification carried out by Lorenita, *et. al* (2013). The difference results with what has been done by Lorenita, *et. al* (2013) because morphological characteristics alone are not sufficient to identify the fungus to the species level appropriately, and molecular identification needs to be acknowledged because the *Trichoderma* species in addition have relatively few morphological characteristics and limited variations that cause species identification errors [6]. For this reason, further identification is needed using the TrichOKEY program.

Molecular identification of LBKURCC40 isolates was carried out in the ITS area using ITS4 and ITS5 primers, and the obtained DNA homology sequence compared to the data contained in GenBank using the BLAST program on NCBI. BLAST analysis was carried out to see the level of sequence homology by comparing the sample sequences of LBKURCC40 fungi isolates in the ITS rDNA area with sequences contained in the database by alignment. Alignment analysis can be done to compare two or more sequences. The results of the homology analysis of sequences from LBKURCC40 isolates are shown in Table 1.

Table 1. Homology identification of the LBKURCC40 isolate sequence

Species name	**Strains**	**GenBank Access Code**	**Reference**	**% Identity of BLAST results**
T. asperellum	Tr266B	KF737411.1	**GenBank**	99%
T. asperellum isolat 2730		EU272534.1	**GenBank**	99%
T. asperellum	T34	EU077227.1	**GenBank**	99%
T. pubescens	DAOM 166162	NR_077179.1	**GenBank**	99%
T. hamatum	CIB T144	EU280105.1	**GenBank**	99%
T. viride		X93981.1	**GenBank**	99%
T. velutinum	TUB F-801	AF149873	[3]	86%
T. fertile	DAOM 167161	AF400260	[3]	89%
T. oblongisporum	CBS 343.93	AF149854	[3]	95%
T. longipile	CBS 340.93	AY865630	[3]	92%
T. stromaticum	CBS 101875	AF098287	[3]	91%
T. spirale	CBS 346.93	AF400262	[3]	89%

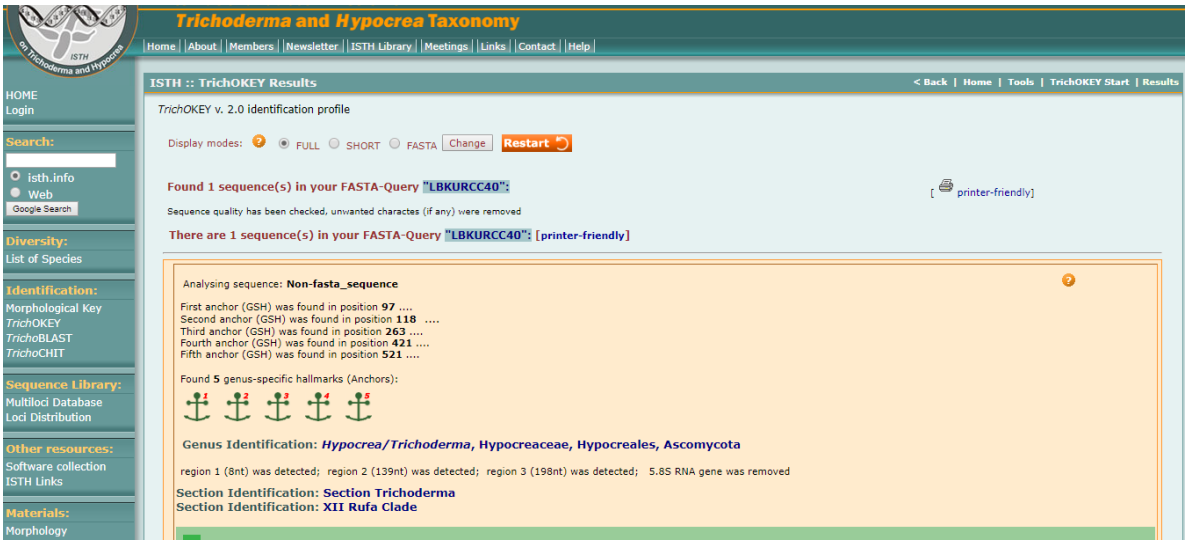
T. ovalisporum	Dis70a	AY380897	[3]	98%
T. erinaceum	DAOM 230015	AY266658	[3]	98%
T. strigosum	CBS 348.93	AY387661	[3]	98%
T. hamatum	DAOM 167057	Z48816	[3]	99%
T. asperellum	CBS 433.97	AY380912	[3]	99%
T. longibrachiatum	CBS 816.68	Z31019	[3]	96%
T. ghanense	G.J.S. 95-137	Z69588	[3]	97%
T. saturnisporum	CBS 330.70	Z48726	[3]	96%
T. sinensis	TUB F-1043	AF486014	[3]	93%
T. effusum	TUB F-354	AF149858	[3]	95%
T. konilangbra	G.J.S. 96-146	AF400261	[3]	96%

Alignment of the BLAST program is carried out statistically by the system by shifting each sequence, and from the number of suitable base pairs. The alignment sequence obtained is used to determine the homology of the sequence being compared. BLAST alignment results in Table 1 show that LBKURCC40 isolates have more than 85% homology with some *Trichoderma* species. The percentage of homology is obtained by comparing the overall amino acid residue equal to the length of the DNA sequences of isolates LBKURCC40.

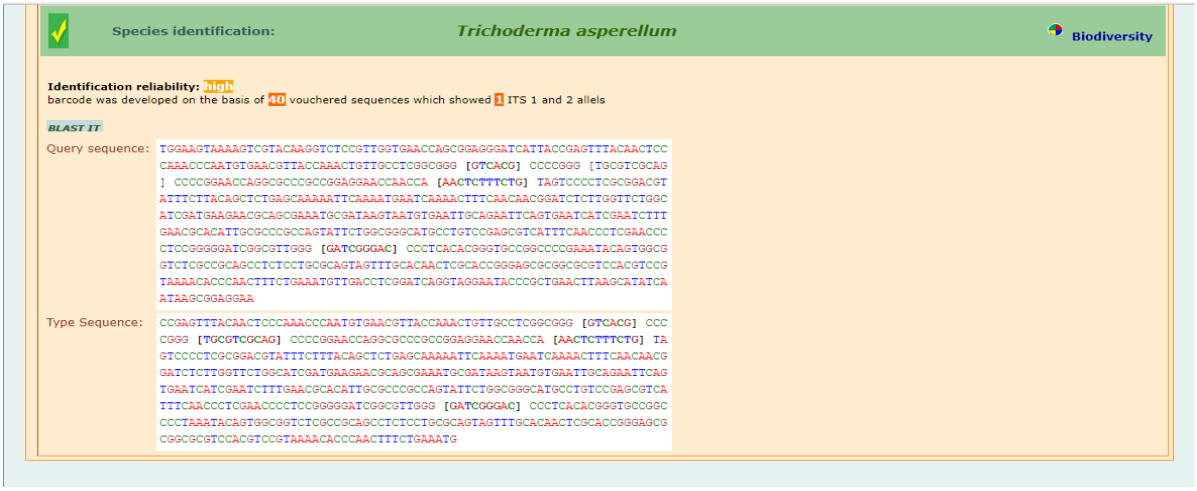
The results of BLAST identification showed that the isolates obtained were indeed *Trichoderma*. Further identification of advanced species was carried out using the TrichOKEY program. TrichOKEY is a Trichoderma molecular identification program at the species level based on DNA BarCode nucleotide DNA [6]. TrichOKEY uses an online method to identify molecules quickly from an isolate at the genus, clade and species level based on a diagnostic combination of several oligonucleotides (special marks) specifically found in ITS1 and ITS2 [4]. The results of the identification of LBKURCC40 isolates using the TrichOKEY program can be seen in Figure 2.

The results of species identification with the TrichOKEY program (Figure 2) show that the species obtained were *Trichoderma*, in accordance with morphological data both macroscopically and microscopically. These results are reinforced by the 5 anchors shown in **Figure 2 (a)** and the species with the closest homology match, namely *Trichoderma asperellum*. These nucleotides among *Trichoderma* species have a very high degree of similarity, making it difficult to distinguish species and frequent species identification errors.

The kinship relationship between fungi at all levels is determined by phylogenetic trees. This difference is due to significant nucleotide differences [5]. Alignment in the filogram tree shape is shown in Figure 2.



(a)



(b)

Figure 2. Identification of LBKURCC40 isolates using the TrichOKEY program

Figure 3 is a filogram tree of LBKURCC40 fungi that shows kinship based on nucleotide changes from evolutionary time. Based on Figure 3, *Trichoderma* LBKURCC40 fungi have the closest kinship to Trichoderma asperellum strain Tr266B fungi with branching number 8558. Bootstrap analysis is carried out with 10,000 repetitions, aimed at testing phylogenetic tree branching and validation. The bootstrap value obtained is more than 70%, which shows that the grouping done is accurate [1].

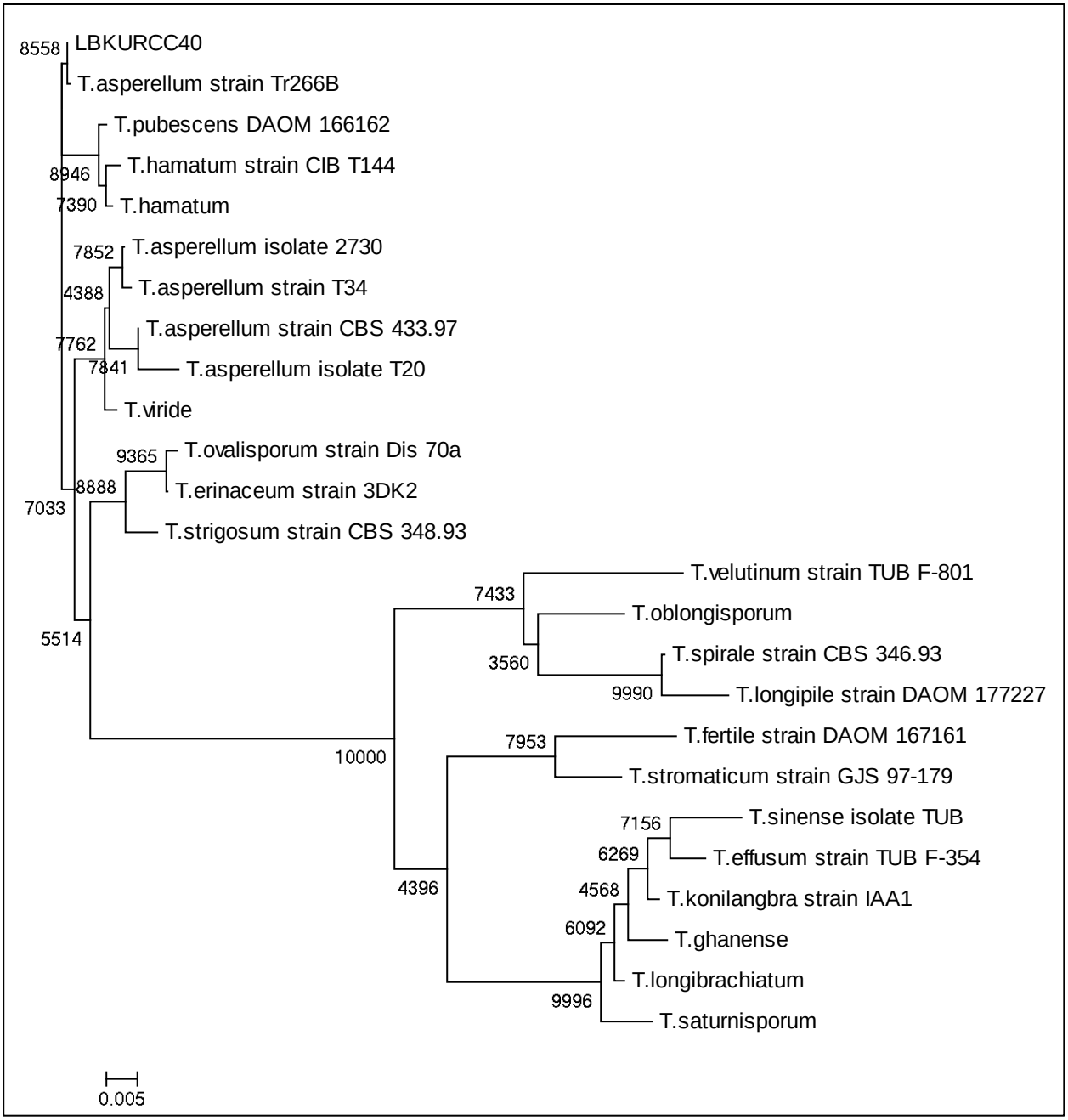


Figure 3. Neighbor-joinin tree (NJ) from the ITS sequence

4. Conclusions

The identification showed that LBKURCC40 isolate was *Trichoderma asperellum* based on morphological and molecular identification characteristics. Phylogenetic analysis with bootstrap 10,000 repetitions at each branch showed endophytic fungus LBKURCC40 had the closest kinship with *Trichoderma asperellum* strain Tr266B. This identification proved that morphological identification could not identify up to the species level, but it needed to be carried out with molecular continued identification.

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