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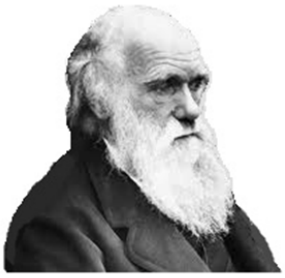

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# Molecular characterization of fungal endophytes associated with *Hypericum perforatum* in Samsun, Turkey

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**ABSTRACT**

In this study, twenty-eight endophytic fungi were isolated from the roots of the good health *Hypericum perforatum* plants collected from Samsun, Turkey. Molecular characterization based on sequence variations in the rDNA-ITS (ITS1-5.8S-ITS2) region of 19 from 28 endophytes obtained from *H. perforatum* was used in genus-level identification. rDNA-ITS sequences were analyzed using the BLAST similarity application on the website of the NCBI. Maximum likelihood rDNA-ITS trees were construct with MEGA11 using the data file of each genus derived from alignments. 13 of the endophytic fungi were determined as *Fusarium* spp., 5 as *Diaporthe* spp. and 1 as *Dactylonectria* spp. To our knowledge, this study was the first report to determine the endophytic fungi in *H. perforatum* plant from Turkey. *Diaporthe* spp. and *Dactylonectria* spp. were determined for the first time in *H. perforatum* in worldwide with this study. Furthermore, *Fusarium* spp. was isolated for the first time from *H. perforatum* in Turkey.

***Keywords:*** *Hypericum perforatum*, Endophytic fungi, rDNA-ITS region, Phylogeny

## 1. INTRODUCTION

Endophytic fungi found in plant tissue maintain a symbiotic relationship with plants without causing any symptoms of infection [1-3]. Endophytic fungi exist in or as part of their

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life cycle by intercellular and intracellular colonization in healthy tissues of host plants. These fungi exist in all plant varieties, such as herbaceous and woody plants, trees, grasses and algae [4]. Many bioactive secondary metabolites produced by endophytes increase the plant's chances of survival and provide protection [5]. Endophytic fungi usually exist without causing damage to the plant but can act as a facultative pathogen when conditions change. These fungi initiate biodegradation of the dead or dying host plant for nutrient recycling [6]. Occurring metabolic interactions between plants and fungi may exert evolutionary pressure on endophytic organisms to synthesize bioactive metabolites. These bioactive metabolites can increase resistance to pests and the fitness of the host plant [7, 8]. Studies on endophytic biodiversity in plants and their bioactive secondary metabolites are increasing daily.

*Hypericum perforatum* (St. John's Wort) is a perennial herb in West Asia, North Africa, Europe, Madeira and the Azores. *Hypericum* includes almost five hundred species and belongs to the Hypericaceae family. *H. perforatum* plants contain medicinally beneficial metabolites. Most can synthesize metabolites with anticancer antioxidant, antiviral, antidepressant, antibacterial and antifungal effects [9]. *H. perforatum* has been a biologically and chemically valuable plant used to treat infectious diseases [10]. Due to these beneficial effects, the use of *H. perforatum*-derived products has increased significantly [11]. Studies with other plants give results showing that many metabolites with different medicinal uses are produced either by the plant, by the endophyte, or by the action of both partners [12].

Endophytic organisms exist in almost all plants [1]. Medicinal plants also contain endophytes that protect them from the host's infectious agents and the ability to survive in adverse environmental conditions. Endophytic fungi in plants have recently received much attention; therefore, their systematic identification and characterization are essential. To date, many endophytic fungi associated with *H. perforatum* plant have been isolated worldwide [7]. *Thielavia subthermophila* was the first endophytic fungus obtained from the roots of *H. perforatum* in by Kusari et al. [13] in India.

This study focused on determining antimicrobial activity, and detailed endophytic fungal characterization still needs to be performed. To date, many endophytic fungi such as *Thielavia subthermophila* [13], *Aspergillus* sp., *Fusarium* spp., *Mucor* sp., *Hypocrea* sp., *Xylaria* sp. [14], *Seimatosporium* sp. [15], *Alternaria* sp., *Cladosporium* sp., *Penicillium* sp., *Scopulariopsis brevicaulis*, *Fusarium poae*, *Trichoderma viride*, *Clonostachys rosea* f. *catenulata* [16], *Aspergillus* sp [17], *Epicoccum nigrum*, *Trichoderma harzianum*, *Alternaria* sp. [9], *Mollisia* sp [18], *Aspergillus terreus* [19], *Acremonium sclerotigenum*, *Plectosphaerella cucumerina*, *Scedosporium apiospermum*, *Plectosphaerella cucumerina*, and *Fusarium oxysporum* [20] have been isolated from *H. perforatum* species. As can be seen, most studies are aimed at determining the effect of drogs, and studies on determining the fungal flora are insufficient. So, the aim of the study was to determined the root fungal flora of *Hypericum perforatum.*

## 2. EXPERIMENTAL PROSEDURES

### 2. 1. Isolation of *Hypericum perforatum*

The good health plants of *Hypericum perforatum* were collected between May and August from different localities (Figure 1) around Samsun: along the roadside, in the stream, in the field and in mountainous areas [21]. Samples placed in plastic bags were labeled placed in plastic bags, labelled and stored at 4 °C until fungal isolation. Endophyte isolation was carried

out within 48 hours after harvest [14]. The roots of *H. perforatum* were washed thoroughly with water, and the specimens were dissected small segments using a sterile lancet. After the parts of the root were sterilized in 1% NaOCl solution for 1 min., they were treated with sterile $dH_2O$ 3 times and placed on sterile blotting paper. Then plant parts were placed on petri dishes containing water agar. The petri dishes were left in the incubater for 2 days at 25 °C in the dark, and then the growth of hyphae was followed daily by light microscopy (40X). Purified fungal isolates were grown in PDA plates [9, 22] and colony colours and sclerotia were determined using the Royal Horticultural Society of London colour chart.

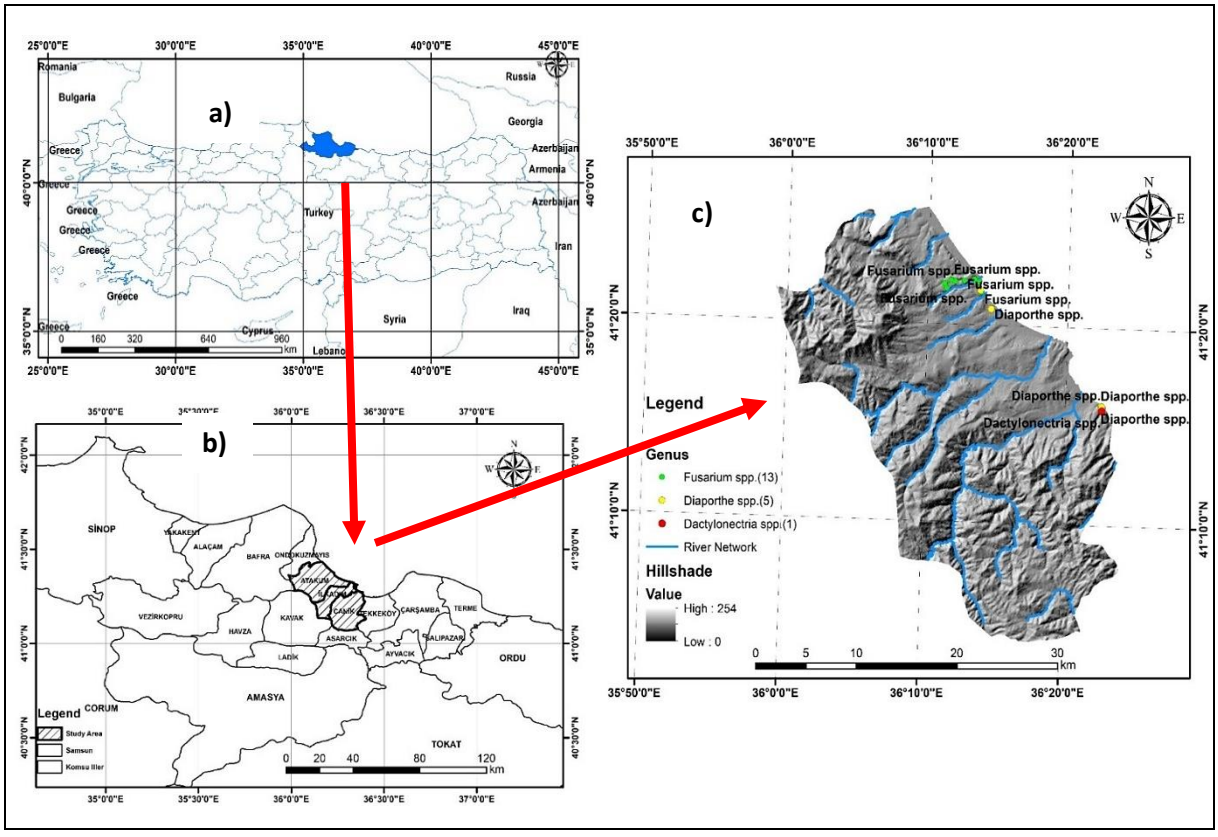


**Figure 1.** Sampling area. **a)** Location of the sampling region in Turkey **b)** Geographical map of Samsun **c)** Geographical map showing endophytic genera isolated from in the study area

## 2. 2. DNA extraction and Amplification of rDNA-ITS

Each endophytic fungal isolate was incubated at 25 °C in the dark in PDB medium (Oxoid) for a week. Then mycelium in the PDB medium was collected and dried with lyophilization during 24-48 hours. Dry mycelium was pulverized and stored at -20 °C in sterile eppendorf [23]. DNA extraction was done using the CTAB method with minor modifications [24, 25]. The rDNA-ITS region were amplified by PCR reaction with the primers ITS-5 (5′GGAAGTAAAAGTCGTAACAAGG3′) and ITS-4 (5′TCCTCCGC TTATTGATATGC3′) [26]. A total volume of PCR mix was applied as 50µl in PCR amplification: 10 ng genomic DNA, 25 pmoles primers (ITS4 and ITS 5), 2.5 mM dNTP mix, 1 U Taq DNA polymerase, 10

µl 5XPCR buffer and 1.5 mM $MgCl_2$. Temperature Profile used in PCR amplification: 3 min at 94 °C (initial denaturation) followed by 1 min at 94 °C, 2 min annealing at 49 °C, 3 min extension at 74 °C for 30 cycles and a 7 min final extension at 72 °C. PCR products stained with Gel Red were run on 1.5 % agarose gel and visualized on UV transilluminator [27].

### 2. 3. Phylogenetic Analysis

Sequencing of the amplified DNA fragments was performed by the Macrogen Company (Macrogen Europe B.V), using the ABI3730XL DNA sequencer. Sequence analysis of the rDNA-ITS regions of the isolates was performed in bi-directionally with the same primers used in PCR reactions. rDNA-ITS sequences were analyzed using the BLAST similarity application on the website of the NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and the genera associated with the isolates were determined according to their similarity values (99-100%). The endophytic fungal isolates from this study along with additional reference and outgroup from GenBank, were aligned using CLUSTALX program [28]. Alignment files manually edited in BioEdit [29].

Maximum likelihood (ML) rDNA-ITS trees were construct with MEGA11 (Tamura et al. 2021) using the data file of each genus derived from alignments. The statistical reliability of the phylogenetic tree was determined with bootstrap (1,000 replications) test using the MEGA11 software [30]. *Leucostoma cinctum* (AC: MH855500 for *Diaporthe* spp.), *Fusarium staphyleae* (AC: MH863667 for *Fusarium* spp.) and *Campylocarpon fasciculare* (AC: MH712264 for *Dactylonectria* spp.) isolates were used as ougroup in phylogenetic trees. The sequence of 19 fungal isolates obtained in our study were deposited in Gen Bank with AC numbers (AC): OQ913885-OQ913903 (Table 1)

## 3. RESULTS

### 3. 1. Identification of Endophytes

As a result of this study, 28 endophytic fungus isolates were obtained from healthy *H. perforatum* plants collected from Samsun, Turkey, in 2018. 19 of these 28 species were identified by molecular techniques. Colony colour and sclerotia of each isolates were determined on PDA using the colour chart of the Royal Horticultural Society of London [23]. The colony colour of D-14 and D-15 (*Diaporthe* spp.) isolates became grey-brown. Different-sized sclerotia grown in the PDA often appeared dense and scattered, embedded in the PDA. Sclerotia diameters range from 0.2 to 0.4 mm, the sclerotia were generally grey-brown. The colony colour of D-19, D-25 and D-28 (*Diaporthe* spp.) isolates became grey-brown. *Diaporthe* spp. produced irregular sclerotia: usually white-brown and 0.2 to 0.4 mm in size on the PDA.

The sclerotia were quite dense and were circularly distributed in the centre and side of the petri dishes. The colony colour of D-6 (*Dactylonectria* spp.) isolate ranged from white to grey-yellow. The diameters of sclerotia varied from 0.1 to 0.4, which were generally white-brown coloured. The sclerotia were very dense and were clustered together and organized in a scattered manner on PDA plate. Except for F-18 isolate (white-orange), the colony colours of other *Fusarium* spp. isolates were similar to each other, having white-pink appearance.

Additionally, the isolates of *Fusarium* spp. produced sclerotia with different size and different distributions on PDA. Sclerotia were usually initially white-coloured, then became grey over time, and diameters of sclerotia ranged from 0.1 to 0.5 mm.

Molecular characterization based on sequence variations in the rDNA-ITS region of 19 of 28 endophytes obtained from *H. perforatum* was used in genus-level identification. Three endophytic fungal genus *Fusarium* spp., *Diaporthe* spp., *Dactylonectria* spp. were determined using BLAST analysis. According to the analysis, 13 isolates were determined as *Fusarium* spp., 5 isolates as *Diaporthe* spp, and 1 isolates as *Dactylonectria* spp. (Table 1). Fragments of approximately 500-700 bp were obtained as a result of PCR amplification of the rDNA-ITS region. Accession numbers of 19 fungal isolates obtained in our study are shown in Table 1. In Molecular characterization of the endophytes, we used NCBI- BLAST algorithm. The endophytic fungal isolates obtained from this study have showed 99% to 100% sequence identity with the corresponding isolates from Genbank.

**Table 1.** Genbank Accession number, GPS location of the sampling points and % similarity of rDNA-ITS region of endophytic fungi.

| Isolate code | GPS Location | Genus | GenBank Accession no | % identity | Accession number of close relatives |
|---|---|---|---|---|---|
| F-3 | 41° 22' 5 N 36° 11' 10 E | *Fusarium* spp. | OQ913885 | 100 % | MH862424 |
| F-4 | 41° 22' 15 N 36° 12' 33 E | *Fusarium* spp. | OQ913886 | 99 % | MH863645 |
| F-5 | 41° 22' 20 N 36° 13' 17 E | *Fusarium* spp. | OQ913896 | 99.05 % | MH855179 |
| F-7 | 41° 22' 12 N 36° 13' 37 E | *Fusarium* spp. | OQ913897 | 100 % | MH855103 |
| F-9 | 41° 21' 47 N 36° 13' 43 E | *Fusarium* spp. | OQ913887 | 100 % | MH864460 |
| F-10 | 41° 21' 47 N 36° 13' 43 E | *Fusarium* spp. | OQ913888 | 99.43 % | MH864013 |
| F-16 | 41° 21' 44 N 36° 13' 46 E | *Fusarium* spp. | OQ913889 | 99.62 % | MH865933 |
| F-17 | 41° 21' 42 N 36° 13' 48 E | *Fusarium* spp. | OQ913890 | 100 % | KX576658 |
| F-18 | 41° 22' 12 N 36° 11' 55 E | *Fusarium* spp. | OQ913891 | 100 % | MH864972 |
| F-20 | 41° 21' 51 N 36° 11' 19 E | *Fusarium* spp. | OQ913892 | 100 % | MH864782 |
| F-21 | 41° 22' 15 N 36°12'33'' E | *Fusarium* spp. | OQ913893 | 99 % | MH865147 |
| F-22 | 41° 22' 12 N 36° 11' 55 E | *Fusarium* spp. | OQ913894 | 100 % | MH855270 |
| F-26 | 41° 22' 16 N 36° 11' 37 E | *Fusarium* spp. | OQ913895 | 99.81 % | MH855035 |
| D-14 | 41° 20' 51 N 36° 14' 32 E | *Diaporthe* spp. | OQ913898 | 99.81 % | KC343174 |

| | | | | | |
|---|---|---|---|---|---|
| D-15 | 41° 21' 47 N<br>36° 13' 43 E | *Diaporthe* spp. | OQ913899 | 99.83 % | KC343174 |
| D-19 | 41° 16' 2 N<br>36° 22' 36 E | *Diaporthe* spp. | OQ913902 | 99.48 % | KC343081 |
| D-25 | 41° 16' 5 N<br>36° 22' 32 E | *Diaporthe* spp. | OQ913900 | 100 % | KC343074 |
| D-28 | 41° 16' 3 N<br>36° 22' 35 E | *Diaporthe* spp. | OQ913901 | 99 % | KC343074 |
| D-6 | 41° 15' 51 N<br>36° 22' 36 E | *Dactylonectria* spp. | OQ913903 | 100 % | MN988721 |

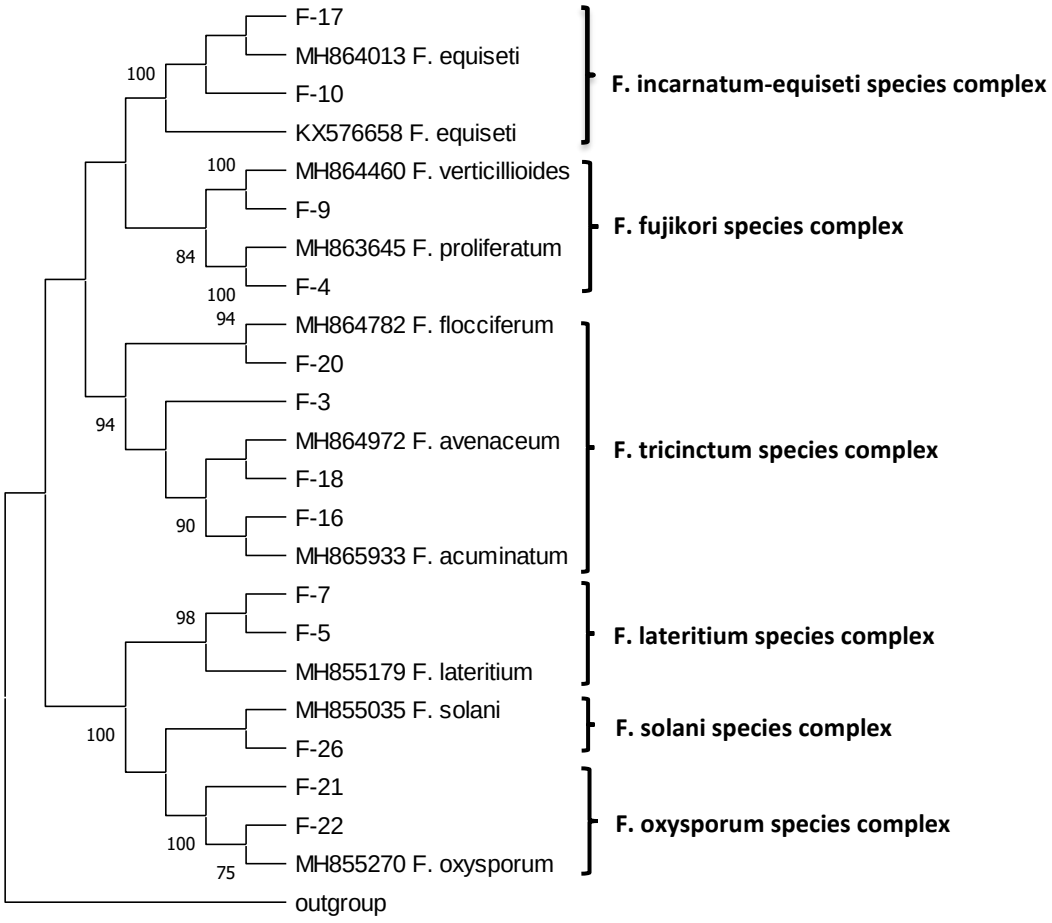


**Figure 2.** Maximum likelihood phylogenetic tree composed of rDNA-ITS dataset of *Fusarium* spp.. *Fusarium staphyleae* was used as outgroup.

Phylogenetic relationships determined by the analysis of rDNA-ITS sequences of endophytic fungi obtained from *H. perforatum* plants were shown in Figures 2, 3 and 4

phylogenetic tree. The maximum likelihood method was used to evaluate the phylogenetic relationship between the samples. The variation between *Fusarium* spp. made according to the base sequences of the rDNA-ITS regions of endophytic fungi are shown in Figure 1. An apparent clustering among *Fusarium* species complexes was observed in the phylogenetic tree obtained by rDNA-ITS nucleotide data. Bootstrap values supported the clustering of different Fusarium spp. in ML tree. It was determined that the isolates F-3, F-16, F-18 and F-20 were associated with *F. tricinctum* species complex, isolates numbered F-4 and F-9 were found to be associated with *F. fujikuori* species complex, isolates numbered F-21 and F-22 with *F. oxysporum* species complex, isolates numbered F5 and F7 with *F. lateritium* species complex, isolates numbered F-10 and F-17 *with F. incarnatum-equiseti* species complex, isolate numbered F-26 with *F. solani* species complex. The variation between *Diaporthe* spp. from endophytic fungi is shown in Figure 2. Isolates of different *Diaporthe* spp. were placed in different clusters in the phylogenetic tree (Figure 3). The ML tree of *Diaporthe* spp. had two major clades, one corresponding to *D. eres* and the other to *D. phaseolorum*. Bootstrap values strongly supported the clustering of distinct *Diaporthe* spp. in ML tree. In ML tree of *Dactylonectria* spp. D6 isolate was clustered in the same branch as *Dactylonectria torresensis* (Figure 4). On the other hand, further studies (using other gene regions) of these isolates are recommended for more reliable distinctions at the species level. As a result of phylogenetic analysis, all fungal isolates were separated into distinct clusters according to their genus.

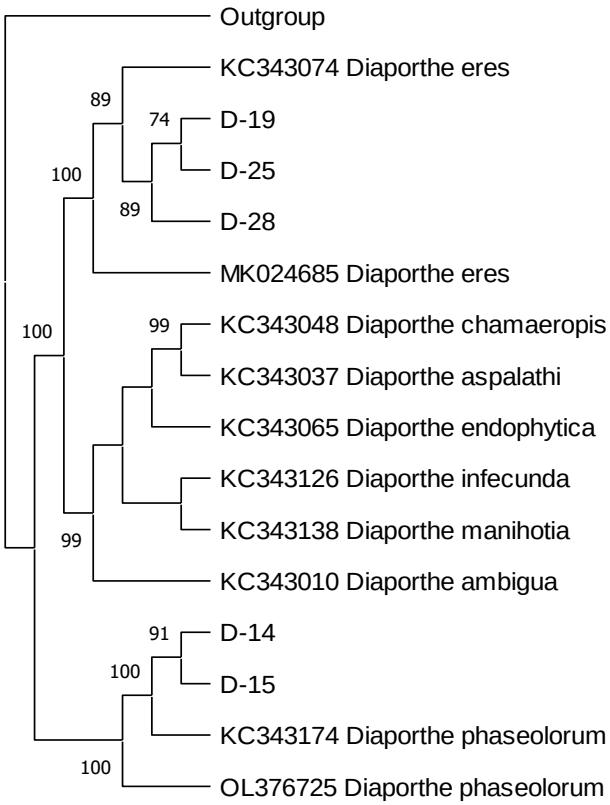


**Figure 3.** A Maximum likelihood phylogenetic tree composed of rDNA-ITS dataset of *Diaporthe* spp.. *Leucostoma cinctum* was used as outgroup.

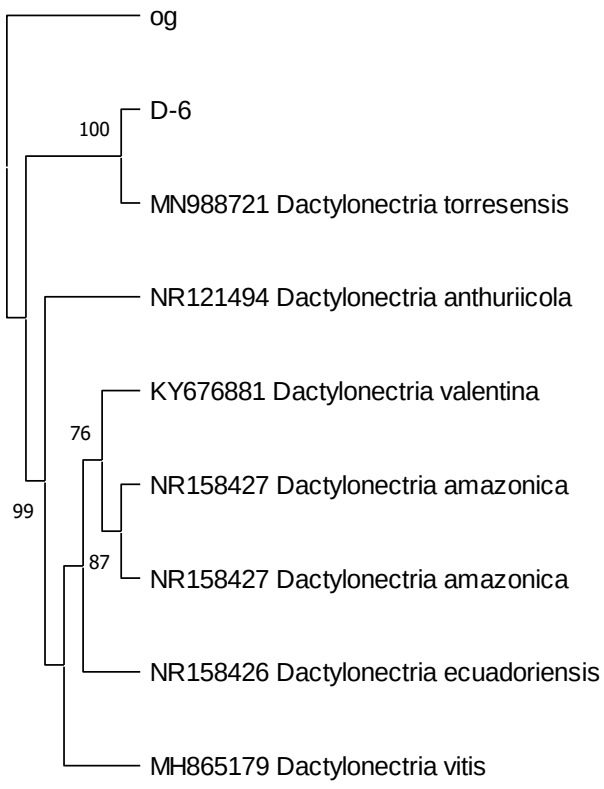


**Figure 4.** A Maximum likelihood phylogenetic tree composed of rDNA-ITS dataset of *Dactylonectria* spp. *Campylocarpon fasciculare* was used as outgroup (og).

## 4. DISCUSSION

The present study focused on the determination of molecular characterization of 19 endophytic fungi isolated from *H. perforatum*. In our study, three genera of endophytic fungi were isolated. It was determined that 13 isolates were *Fusarium* spp., 5 isolates as *Diaporthe* spp. and 1 isolates as *Dactylonectria* spp. (Table 1). They were observed that most of the isolates that were characterized in our study belonged to *Fusarium* species. The dominant fungus in root endophyte was *Fusarium* spp. at the genus level in our study. Phylogenetic analyses based on the sequence of rDNA-ITS revealed that different endophytic fungal isolates were in distinct clusters (Figs. 2, 3 and 4). The Maximum likelihood tree generated with the ITS sequences showed an apparent clustering among endophytic fungus species. Bootstrap rates have supported the clustering of different species in all three phylogenetic trees.

An endophytic fungus was isolated from the roots of *H. perforatum* for the first time in India. Endophytic fungus was identified as *Thielavia subthermophila* by morphological and molecular methods [13]. Similarly, in a study conducted in China, a total of 21 endophytic strains were isolated from *H. perforatum* and preliminary classified into 5 genera, including *Fusarium* spp., *Mucor* sp., *Aspergillus* sp., *Xylaria* sp. and *Hypocrea* sp. which were the dominant strains.

They studied the isolation and antimicrobial screening of endophytic fungi isolated from *H. perforatum*. The study first focused on investigating the isolation and antimicrobial screening of endophytic fungi isolated from *H. perforatum* [14]. Another research was carried out in Canada. This research was the first fungal isolation from *H. perforatum* plant leaves [15]. In our study, endophytic fungi were obtained from the roots of the *H. perforatum* plant. In another study in Uzbekistan by Egamberdieva et al. [31], endophytic fungi *Fusarium oxysporum* and *Alternaria alternata* were also isolated from the *H. perforatum* plant. As can be seen, many endophytic fungal isolates have been obtained from the *H. perforatum* plant from many parts of the world until today. However, according to literature, there is no study the endophytic fungi related to *H. perforatum* plant from Turkey. In this sense, our study is the first report to determine the endophytic fungi in the *H. perforatum* plant in Turkey.

Although many endophytic fungal isolates have been obtained from the *H. perforatum* plant in studies conducted in different parts of the world, this study is the first report on the isolation of endophytic fungi *Diaporthe* spp. and *Dactylonectria* spp. from *H. perforatum* in worldwide. *Diaporthe eres* was previously isolated from *H. annulatum* and *H. kouytchence* by Henzelyova et al. [20]. But in present study, *Diaporthe* spp was determined for the first time *in H. perforatum* Worldwide. In this study, D-6 isolate was found to be associated with *Dactylonectria torresensis*. *D. torresensis* was first described as *Ilyonectria torresensis* [32].

Later *Ilyonectria torresensis* was renamed as *Dactylonectria torresensis* after reassessment [33, 34]. Considering this situation, the presence of *Ilyonectria torresensis* isolated from *H. perforatum* before 2014 [33] was investigated. There was no record of the *Ilyonectria* species obtained from *H. perforatum*. As a result, it was confirmed that *Dactylonectria* spp. was reported for the first time in worldwide from *H. perforatum* plants. In addition, in other studies conducted in worldwide, endophytic fungi belonging to the genus *Fusarium* were obtained from the *H. perforatum* plant, but in this study, *Fusarium* spp. endophytes from the *H. perforatum* plant in Turkey were determined for the first time.

An endophytic fungus was first isolated in 2008 from the stems of the medicinal plant *Hypericum perforatum* by Kusari et al [13]. After this date, as described above, a significant increase has been observed in studies on the *H. perforatum* plant. Studies have mainly focused on identifying secondary metabolites produced by endophytic fungi. These compounds are significantly affected by the diversity of endophytic fungi that live with the plant. Endophytic fungi in medicinal plants have recently received much attention, so their systematic identification and characterization are essential. Thanks to this study, new endophytic fungi associated with the *H. perforatum* were identified worldwide and contributed to literature. The new endophytic fungal species we determined in the *H. perforatum* plant in our study may contribute to discovering new secondary metabolites associated with the plant in future studies.

## 5. CONCLUSION

In our study, endophytic fungi have been isolated from root tissues of the *H. perforatum* plants collected from Samsun, Turkey. This study is the first report to determine the endophytic fungi in the *H. perforatum* plant in Turkey. Molecular characterization was carried out based on sequence variations in 19 rDNA-ITS regions from 28 endophytes obtained from *H. perforatum*. Our study showed that 13 of endophytic fungi were characterized as *Fusarium* spp, 5 as *Diaporthe* spp and 1 as *Dactylonectria* spp.

In present study, *Diaporthe* spp. and *Dactylonectria* spp. were determined for the first time *in H. perforatum* Worldwide. In previous studies in the world, the record of the existence of these endophytic fungi was not available in *Hypericum perforatum*. However, Fusarium spp. was also isolated from *H. perforatum* in Turkey for the first time.

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