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# Molecular Identification of *Alternaria alternata* (Fr.) Keissl. from the Leaf Blight Disease of *Centella asiatica* L.

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## ABSTRACT

**_Centella asiatica_ (L.), frequently known as Thankuni, is an important ethnobotanical plant in Bangladesh. This study was conducted to evaluate the morphological characteristics, cultural factors and molecular identification of the causal agent of _Alternaria_ leaf blight disease of _C. asiatica_. The potato dextrose agar (PDA) medium recorded the maximum mycelial growth (69 mm), followed by the yeast extract agar (YEA) medium, while the honey peptone agar (HPA) medium recorded the lowest growth (27 mm). The optimal pH and temperature for mycelial growth of _Alternaria alternata_ were 6 and 30˚C, respectively. Internal transcribed spacer (ITS) region of _Alternaria alternata_ PCR products measured 558 bp and blast search showed 99% sequence similarity with _Alternaria alternata_ species complex. To the best of our knowledge, _Alternaria_ leaf blight disease caused by _Alternaria alternata_ is the first record in Bangladesh.**

## 1. INTRODUCTION

*Centella asiatica* (L.) is a small perennial herb, commonly known as a shrimp plant [1]. It may be found practically everywhere in the world and grows abundantly in gloomy, swampy, damp, and wet areas like paddy fields and river banks, creating a thick green carpet. The leaves, which have seven veins and are edible, are reniform, orbicular or oblong-elliptic in shape, thin, alternate, and yellowish-green in color. The plant spreads out horizontally through its subterranean roots and green to red stolons that connect to one another [2]. The use of *Centella asiatica* is becoming more and more popular, particularly for therapeutic purposes. *Centella asiatica* has long been recognized for its extensive medicinal potential. It has been used historically for the treatment of diseases like hepatitis, measles, toothaches, syphilis, leucorrhoea and diarrhea [3]. Compared to the petioles and the roots, the leaves had the highest concentration of phytochemicals. Niacin carotene, vitamin A, vitamin B1, vitamin B2 and vitamin C are also abundant in it [4]. Some of the significant bioactive chemicals that are responsible for its antioxidant, antimicrobial, antiulc-

er, antifilarial, antiviral, and numerous other actions are madecassic acid, asiatic acid, $\alpha$-terpinene, $\alpha$-copaene and $\beta$-caryophyllene [3]. Presently these medicinal herbs are commercially cultivated in the Northern part of Bangladesh, while fungal diseases are major threats to the commercial cultivation of *C. asiatica*.

Due to their susceptibility to various infectious microbes that cause qualitative and quantitative losses, plants yield less as a result. Several fungi that cause disease in the *C. asiatica* plant worldwide are *Pseudocercospora centelli* [5], *Cercospora centellae* [6], *Fusarium equiseti* [7] etc. which significantly damage the plants on cultivated land. The roots have the greatest variety of endophytic fungi, which are found in the leaf, stolon and petiole of the *C. asiatica*. The most prevalent endophytic fungi in plants are *Colletotrichum destructivum*, *Fusarium solani*, *Ceratobasidium* sp., *Alternaria* sp. and *Curvularia* sp. Shukla *et al.* [8] reported that different biotic hindrances that affect medicinal plants frequently result in yield and economic losses as well as changes to the quality of the goods that are made from the plant. One of the most dangerous fungal pathogens is the *Alternaria* spp., which is found in medicinal plants all around the world.

Phylogenetic relationships between and within populations of several *Alternaria* species have been inferred using molecular markers such as small subunit ribosomal DNA as well as sequence data from ribosomal DNA and other protein-coding genes [9]. The ITS region of genomic DNA is the most beneficial of these methods for determining the phylogenetic relationships amongst fungi. The rDNA's ITS region is one of the more changeable regions.

The growth, development and sporulation of fungus depend on the culture medium. In addition, important environmental elements like temperature, humidity, pH and others are necessary for the germination of fungal spores, the growth of germ tubes and ultimately the development of disease. These research materials thus provide important information about the proper identification of pathogenic fungi of *Centella asiatica* in Bangladesh. Therefore, the present research work was undertaken to identify and appraise *Alternaria alternata* from leaf blight disease of *Centella asiatica* by morphological and molecular characteristics based on ITS region.

## 2. MATERIALS AND METHODS

### 2.1. Sample Collection

*Centella asiatica* L. leaf blight was found in many locations on the campus of Jahangirnagar University, Savar, Dhaka, Bangladesh and in the Manikgonj district of Bangladesh. To isolate and identify the causal organism of the collected diseased samples were packaged individually in sterile plastic bags. For further study, these samples were subsequently preserved in the Laboratory.

### 2.2. Isolation and Morphological Characterization of the Fungus

The tissue planting technique was used to extract the fungus from the *C. asiatica* leaf blight symptoms. Infected sections of *C. asiatica* were cut into 0.5 cm length pieces and were intended to contain both fungal-infected and non-infected tissues. After that, the items were sterilized for 3 minutes with a NaOCl (5%) solution before being repeatedly washed with distilled water. Four samples were put into a PDA medium and cultured for ten days at a temperature of 25˚C with 12/12 hours of darkness and light subsequently. The growing mycelium was transferred to fresh PDA plates and PDA slants in order to create a pure culture. The pure culture of the isolated fungus was identified microscopically using traditional methods based on colony size, shape, mycelium, and conidiophore [10].

### 2.3. Culture Media, Temperature and pH on the Growth of Fungus

Eight different culture media, including sucrose glucose agar (SGA), yeast extract agar (YEA), honey peptone agar (HPA), maltose agar (MA), carrot agar (CA), potato dextrose agar (PDA), honey agar (HA), and potato sucrose agar (PSA), were prepared to measure the mycelial growth of the isolated fungus ac-

cording to Alam *et al.* [11]. To determine the optimal temperature for the mycelial development of isolated fungus, different levels of temperature conditions (15˚C, 20˚C, 25˚C, 30˚C, and 35˚C) were evaluated. For the study of the effect of pH, the PDA medium was accustomed to pH 5, 6, 7, 8, and 9 with the help of a digital pH meter and the addition of 1 N NaOH or lactic acid to adjust the pH and then autoclaved. After autoclaving, media was poured into the Petri plates and 2 mm diameter agar blocks containing tested fungus from a 7-day cultured PDA media were positioned in the center of each agar plate and it was then incubated at 30˚C for 7 days. In accordance with Alam *et al.* [11], the radial development of mycelia on each petri dish was assessed in three directions.

### 2.4. Molecular Characterization

Molecular characterization was carried out after microscopic inspection with a commercial service provided by Invent Technology, Dhaka, Bangladesh. The Maxwell Cell Kit (AS1030, Promega, USA) was used to extract genomic DNA from samples of fungi. The PCR experiment employed the primers ITS4 (5'-TCCTCCGlCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAA AGTCG TAACAAGG-3') [12]. In a 25-µl reaction mixture with an LA Taq (TAKARA BIO INC, Japan), the PCR reaction was carried out using 20 ng of genomic DNA as the template. Taq polymerase was activated for 1 minute at 94˚C, followed by 35 cycles of 94˚C for 30 seconds, 55˚C for 30 seconds, and 5 minutes at 72˚C, with a 10-minute step at 72˚C for final extension. A 1 kb DNA ladder was used as a size marker as the amplified PCR products were electrophoresed on a 1.5% agarose gel in 1 TAE buffer for 1 hour at 100 V, and then stained while being stirred in an EtBr solution (0.5% g/mL). A UV transilluminator was used to see and photograph the stained gels. The amplification products were purified using the Maxwell® 16 DNA Purification Kits (Promega, USA). First BASE Laboratories SdnBhd in Malaysia conducted a bidirectional sequencing analysis on the purified PCR products.

BioEdit and MEGA6 were used to verify DNA sequences. The NCBI received the sequencing data under accession number JUF0039. The ITS sequences were utilized in a BLAST search to identify the closest-matching taxa. MEGA6 was used to perform multiple sequence alignments. Clustal W was used to convert the data from fasta to MEGA format. The Akaike Information Criterion (AIC) was used to identify the evolution models. Tamura-3 parameter was the model used for investigation. The robustness of the branches was assessed using 1000 bootstrap repetitions and a max-trees setting of 1000 after performing maximum likelihood (ML), neighbor-joining (NJ), and maximum parsimony (MP) analyses. The halting condition was used to infer the number of replications. According to Tamura *et al.* [13], bootstrap values better than 60% were acceptable.

### 2.5. Statistical Analysis

Standard statistical analysis tools, such as MS Excel, SPSS 16.0, MEGA 11.0 program, and BLAST tool, were used to analyze the data produced during the research work. Data were analyzed using one-way ANOVA with Duncan's post-hoc test in SPSS-16.

## 3. RESULT AND DISCUSSION

### 3.1. *Alternaria* Leaf Blight Disease of *Centella asiatica*

*Alternaria* blight is one of the most dominant fungal diseases of *Centella asiatica*. The appearance of irregular, frequently round, brown to dark brown leaf patches with concentric lines inside the spots is one of this disease's symptoms. Leaf blight is frequently caused when the circular spots combine to create substantial areas (**Figure 1**). Numerous times, delicate twigs and pods will have little black patches as well. In terms of morphology, *Alternaria* creates a series of concentric rings around the initial site of attack on the host leaf. The symptoms first emerged as irregularly shaped patches that ranged in color from light brown to dark brown to black and were surrounded by necrotic hallow all over the leaf blade. The top and middle leaves were gradually affected by the illness.

Figure 1. *Alternaria* leaf blight disease of *Centella asiatica*.

### 3.2. Morphology of *Alternaria alternata*

The spores have the widest base and progressively narrow to an elongated beak. The production of polymorphous conidia, either individually or in short or long chains, with transverse, longitudinal, as well as oblique septa and longer or shorter beaks, is a distinctive feature of the species. These polyphagous fungi's spores are frequently found in the soil and the atmosphere. Most *Alternaria* species have conidiophores that generate asexual conidia that are between 160 and 200 μm long [14]. Myceliums were found with branching, septate, sub-hyaline and smooth to verruculose hyphae that are either surface or submerged-level. Conidiophores are typically solitary, brown, macro-nematous, simple or branching or with one to numerous geniculations, or more ellipsoidal or ovoid conidia in a single hypha (**Figure 2**).

### 3.3. Growth Characteristics of *Alternaria alternata*

**Figure 3** and **Figure 4** have been showed the impact of several culture media, including SGA, CA, PSA, YEA, PDA, HA, HPA, and MA, on the mycelial development of *Alternaria alternata*. According to the findings, the PDA medium had the maximum mycelial growth of *A. alternata* (69 mm), followed by the YEA medium, while the HPA medium had the lowest growth (27 mm).

Several mycologists reported that PDA is one of the ideal and most frequently used culture medium due to its straightforward composition and capacity to support the mycelial growth of fungi [15, 16]. The results of the current experiments support those of earlier researchers who conducted physiological research on the best conditions for *Alternaria* sp. growth and sporulation. *Alternaria alternata* was grown on Potato Dextrose Agar (PDA), V8 Juice Agar (V8 Agar), and Mycological Agar (MA) media and results showed that PDA and V8 agar were the two best culture media for sporulation [17]. According to Pradeep *et al.* [18], PDA (74.96 ± 0.35 mm) had the highest radial mycelial growth, followed by Malt extract Agar (74.03 ± 0.25 mm) and Oat Meal Agar (74.03 ± 0.15 mm). The growth of *Alternaria carthami* was tested on eight culture mediums. Results showed that potato dextrose agar (90.00 mm), which considerably provided the maximum growth was followed by potato malt agar (84.16 mm) with good sporulation [19]. The PDA was the optimal medium for the mycelial growth of isolated fungi, according to this study.

The mycelial growth of the isolated pathogen was tested based on temperature and their results have been presented in a graphical presentation where the mycelial growth of the isolated pathogen was measured after 7 days of inoculation. The experimental plates were incubated at five different temperatures viz., 15˚C, 20˚C, 25˚C, 30˚C and 35˚C and the results have been shown in **Figure 5**. The current study looked at how temperature affected the radial mycelial development of *Alternaria alternata* on a PDA medium in an *in vitro* environment. According to the findings, *A. alternata* grew at a rate that was maximum at 30˚C and then 25˚C. The highest mycelia growth and sporulation of *Fusarium solani* were recorded at 30˚C in our experiment, and a sharp decline in mycelial growth and sporulation was seen at 30˚C, which is similar to the findings of Ahmmed *et al.* [20].

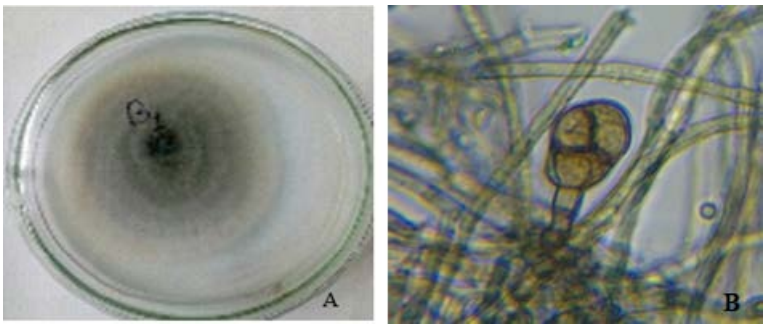


Figure 2. (A) Mycelial growth of *Alternaria alternata* on PDA medium; (B) Microscopic view of mycelium and conidia of *Alternaria alternata* (40×).

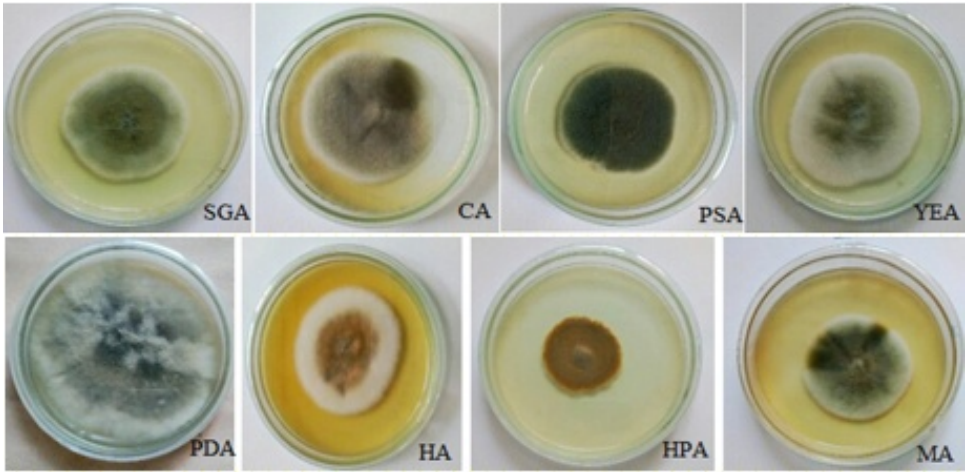


Figure 3. Effect of different culture media on mycelial growth of *Alternaria alternata*. SGA, sucrose glucose agar; CA, carrot agar; PSA, potato sucrose agar YEA, yeast extract agar; PDA, potato dextrose agar; HA, honey agar; HPA, honey peptone agar; MA, maltose agar.

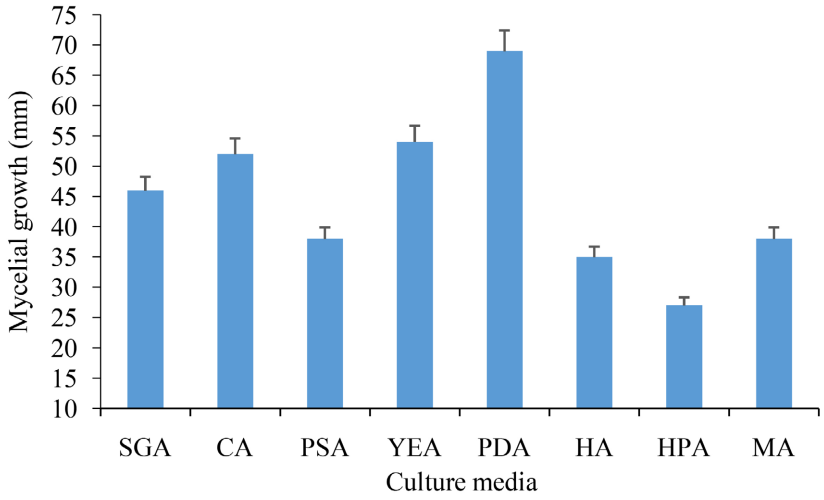


Figure 4. Effect of different culture media on the mycelial growth of *Alternaria alternata* at 7 dpi. SGA, sucrose glucose agar; CA, carrot agar; PSA, potato sucrose agar YEA, yeast extract agar; PDA, potato dextrose agar; HA, honey agar; HPA, honey peptone agar; MA, maltose agar.

Although pH is a crucial factor for understanding the ecology of rotting fungus, the experimental plates of this work were incubated at four different pH levels, namely 5, 6, 7, and 8. *Alternaria alternata* showed the maximum mycelial growth (43.6 mm) at pH 6, whereas pH 8 showed the lowest mycelial growth (12.2 mm) (**Figure 6**). To determine the impact on the growth of *Alternaria carthami*, nine pH values were investigated. The results showed that excellent sporulation led to the largest mean mycelial growth (85.83 mm) at pH 6.5, which was followed by excellent and good sporulation at pH 6 (82.00 mm) and pH 7 (70.33 mm), respectively. According to Singh [21], *A. brassicae*'s optimal temperature for development and sporulation was 22˚C, while its optimal pH was 5.5. However, under *in vitro* circumstances, the effects of various pH values were evaluated on the growth of *A. alternata*. The experiment's findings showed that the pH range of 6.00 to 6.50 was ideal for *A. alternata* growth [22].

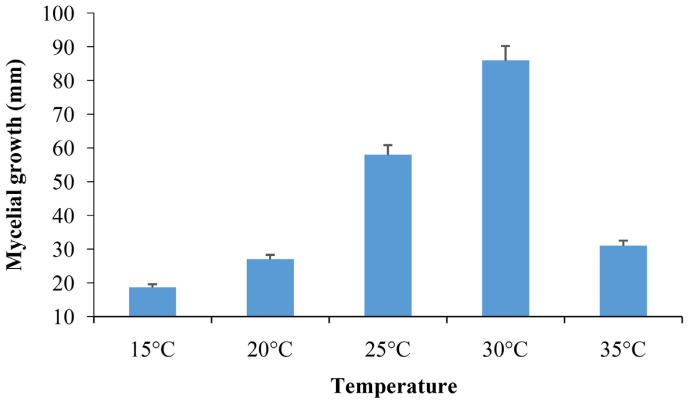


**Figure 5.** Effect of different temperatures on the mycelial growth of *Alternaria alternata* at 7 dpi.

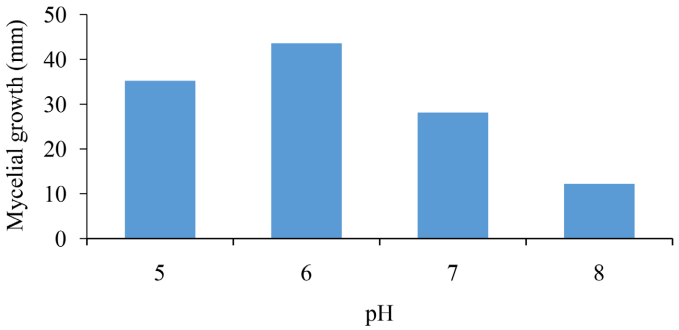


**Figure 6.** Effect of different pH on the mycelial growth of *Alternaria alternata* at 7 dpi.

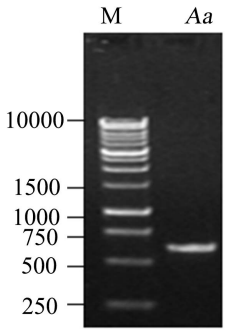


**Figure 7.** PCR products of the ITS region of *Alternaria alternata* (*Aa*). M, molecular size marker (10 kb DNA ladder); lane *Aa*, *Alternaria alternata*.

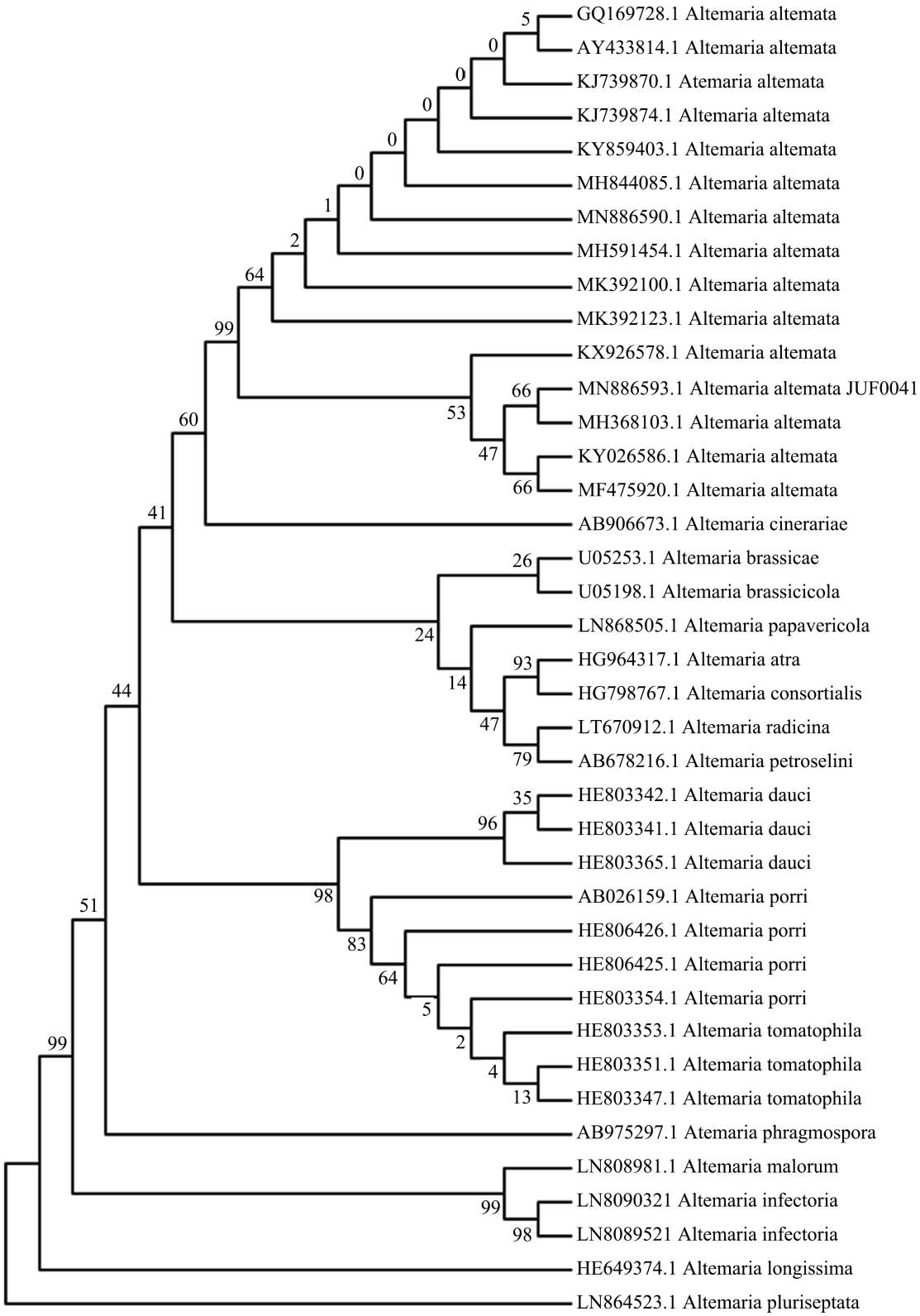


**Figure 8.** **Maximum likelihood tree was created using bootstrap value (Bootstrap replication = 1000) and analysis of the ITS sequencing dataset of the investigated organism. JUF0041 is a marking on our organism (MN886593.1).**

### 3.4. Molecular Identification of *Alternaria alternata*

Fungal rDNA's extremely varied ITS regions are crucial for using PCR to identify between different species of fungi. Using ITS4 and ITS5 primers, the 558 bp-long ITS region was amplified for molecular identification before being sequenced. However, other species in the same genus have shorter ITS regions (**Figure 7**). The internal transcribed spacer region of *Alternaria alternata* was quantified by gel electrophoresis of PCR products produced by primers ITS4 and ITS5. As a result, this characteristic may help differentiate *Alternaria alternata* from other species in the same genus. The ITS region of genomic DNA has recently been shown to be extremely helpful for identifying fungi at lower taxonomic levels through molecular phylogenetic analyses. According to Alam *et al.* [11], the ITS region of rDNA varies between species and even between strains.

Using ITS4 and ITS5 primers, the ITS region was amplified and sequenced (**Figure 8**). From the NCBI database, 39 fungal species were chosen for phylogenetic analysis based on the nucleotide sequences of the ITS sections. Six distinct clusters were discovered in the phylogenetic tree using maximum parsimony. A previously found fungus, KX926578 and the rDNA sequence of the ITS region (MN 886593.1) were evaluated for percent homology. Reciprocal homologies of the ITS region sequences 99%. The results indicated that all the individual species of *Alternaria alternata* belong to the major cluster. Using common single primer pairs in PCR, the ITS region may be quickly and readily amplified. High levels of similarity between identical ITS sequences and genetic distance were observed. These strains may be easily distinguished from one another based on the ecological distribution, substitution and insertion or deletion polymorphisms of the base position [11, 23]. The size variation was brought on by changes in the number of nucleotides. According to Alam *et al.* [24] ITS sequences fluctuate between species in a genus but are genetically stable or exhibit little change within the species. The great genetic compatibility among the strains examined and the efficient gene flow within groups are likely the causes of the genetic diversity found within groups. Results indicated that the *Alternaria* leaf blight disease of *Centella asiatica* was caused by *Alternaria alternata* and molecular evidence results also supported and confirmed the finding result of this study.

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

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