

## MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF ENDOPHYTIC FUNGI ISOLATED FROM *ZINGIBER OFFICINALE* ROSC.

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### Abstracts

This study was conducted to discover wide spectrum of endophyte diversity from the *Zingiber officinale* Rosc. Endophytic fungi were obtained from different plant tissues. All the isolated strains were identified up to genus level following described colony morphology. The recognized five different morphotypes were subjected to sequence analysis of internal transcribed spacer (ITS) gene. *Fusarium proliferatum*, *Fusarium solani* and *Cladosporium cladosporoides* were identified based on comparison of the BLAST results and phylogenetic identification. The presented study provides the comprehensive explanation of the interrelation of morphological and molecular homologies for the identification of the prospective fungi.

### Introduction

Endophytic fungi are miscellaneous polyphyletic groups of microorganisms that can boom asymptotically in different healthy tissues (stems, leaves, and/or roots) of living plants. It is estimated that over one million endophytic fungal species occur in the nature (Faeth and Fagan, 2002). The bioactive compounds produced by endophytic fungi can induce the production of a plethora of known and novel biologically active secondary metabolites that can be utilized and functional by human as important medicinal resources (Zhang *et al.*, 2006; Firková *et al.*, 2007; Rodriguez *et al.*, 2009).

*Zingiber officinale* Rosc. (Ginger) is a herbaceous perennial plant of Zingiberaceae family. The family Zingiberaceae has 52 genera and 1400 species. It is distributed throughout tropical Africa, Asia, and the Americas. It has great traditional medicinal value being employed in many indigenous medical systems since ancient time. Many members of Zingiberaceae are used in Ayurvedic, Unani, and Homoeopathic systems of medicine. That is why this family is ethnopharmacologically important. Phytochemical investigation of the rhizomes of several *Zingiber* sp. has disclosed the presence of bioactive compounds such as gingerols, shogaols, diarylheptanoids, phenylbutenoids, flavanoids, diterpenoids and sesquiterpenoids (Sivasothy *et al.*, 2011). The gingerols are identified as the major active components in the fresh rhizome of the plant. In addition, shogaols, dehydrated gingerol derivatives, are the predominant pungent constituents in dried ginger (Jiang *et al.*, 2006). This plant also reported to possess several pharmacological activities such as antimicrobial activity, anti-diabetic activity, nephroprotective

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activity, hepatoprotective activity, larvicidal activity, anticancer activity, analgesic activity, anti-inflammatory activity, immunomodulatory activity, antioxidant activity, anthelmintic property etc. (Kumar *et al.*, 2011). But searching of endophytic fungal miscellany from this plant remains unacquainted.

Endophytic fungi show diversity that belongs to several taxonomic groups. Taxonomy uses hierarchical classification as a way to facilitate scientists understands and organizes the diversity. For the taxonomical identification, both the morphological and the molecular characteristics of the organism are needed. Morphological identification is the conventional method of describing physical features of the filamentous fungi that are given some possible clues of identification. Due to the drawbacks of conventional methods, molecular techniques are used to investigate the problems related to identification and classification of species. Identification of fungi to species level is vital for both basic (ecology, taxonomy) and applied (genomics, bioprospecting) applications in scientific research. As for genetic materials, phylogenetic analysis is another alternative way of species identification. It is the study of evolutionary development of a gene to understand the evolutionary relationships among species. Thus, in this report a detailed characterization of endophytic fungi isolated from *Zingiber officinale* had been described with respect to morphological and molecular approach for further preliminary screening of their bioactive potentiality.

## Materials and Methods

### Plant collection and isolation of endophytes

Healthy and mature *Z. officinale* plants were collected from Dhamrai, Dhaka, Bangladesh and the plant was recognized and validated by a taxonomist of Bangladesh National Herbarium (BNH), Dhaka, Bangladesh. A receipt herbarium specimen under the accession number DACB 55762 of *Z. officinale* was assigned and deposited at the BNH.

Fungal endophytes were isolated from leaf, bark and petiole parts of the fresh and healthy plant tissues following a revised surface sterilization method (Chowdhury *et al.*, 2016; Khan *et al.*, 2016). For surface sterilization, the selected cleaned plant parts were undergone into a small cutting (2–3 cm) over a sterile glass plate. The cutting edges were then subjected to subsequent treatment with 70% ethanol, 1.3 M sodium hypochlorite and finally sterile distilled water respectively. The treated plant samples were then soaked on sterile filter paper and placed in an antibiotic (streptomycin 100 mg/l) containing water agar medium for incubation (at  $28 \pm 2^\circ\text{C}$ ). The visible mycelium come out over 4-6 weeks was further transferred into Potato Dextrose Agar (PDA) medium to isolate the endophytes by comparing the growth of the exophytes incubated in a same manner collected from the unsterilized plant segments to be used for control study. The isolated pure cultures were cultivated on PDA medium and for obtaining the crude fungal extracts of each isolate, the cultured medium of all the fungal strains were extracted three times with ethyl acetate.

### Identification of the isolated endophytes

*Morphological identification:* Isolated endophytes were identified morphologically based on macroscopic and microscopic features. Morphological characteristics such as growth pattern, hyphae structure, the color of the colony and medium, aerial mycelium, surface texture, sporulation and production of acervuli, the size and coloration of the conidia were examined in 3rd, 6th, 9th and 12<sup>th</sup> days of cultural growth on PDA medium until full growth of fungi and compared with the standard taxonomic key (Devi and Prabakaran, 2014; Barnett and Hunter, 1972). Microscopic study of the isolated strains was done followed by staining with lactophenol

cotton blue (LPCB) and examined under a bright-field and phase contrast microscope (Kruss, Germany) with objective lens of 40 times magnification and 0.65 numerical apertures (Sadananda, 2014).

### **Molecular identification**

To identify the species of the respective fungus, selected endophyte isolates were subjected to molecular characterization by DNA amplification and sequencing of the internal transcribed spacer (ITS) region. Here ITS4 (5'-TCCGTAGGTGAACCTGCGG-3') (Invitrogen, USA) and ITS5 (5'-TCCTCCGCTTATTGATATGC-3') (Invitrogen, USA) were used as forward and reverse primer, respectively (White *et al.*, 1990). Fungal DNA isolation was carried out by using DNeasy Minikit (QIAGEN, USA) according to the manufacturer's protocol. The target DNA sequence was then amplified by polymerase chain reaction (PCR) using Hot StarTaq Master Mix Kit (QIAGEN, USA). ITS4 and ITS 5 primers, were mixed with Hot Star Taq Master Mix Kit and DNA template in a total volume of 50µL where each PCR reaction mixture contained 5-10 ng of genomic DNA, 1 µM each of the primers ITS4 and ITS5 and 1 U of Hot Star Taq Polymerase. The mixture was then applied to the thermal cycler (BioRad, USA) for 35 cycles using initial pre-heat at 95°C for 2 minutes; denaturing for 1 minute at 95°C, annealing for 40 seconds at 56°C, extension for 1 minute at 72°C, final extension for 10 minutes in 72°C. Approximately 550 bp PCR product purification was carried out by using perfect Prep Gel Cleanup Kit (Eppendorf, USA) following manufacturer's protocol. The amplified pure fungal DNA (PCR product) was sequenced using electrophoretic sequencing on an ABI370X1 DNA analyzer (Applied Biosystems, USA) using Big dye Terminator v 3.1 cycle sequencing kit.

### **Phylogenetic analysis**

The resulting sequences of the isolated endophytes were then subjected to nucleotide BLAST in order to compare the regions of similarity of the query sequences against the deposited biological sequences into the NCBI databases. To understand the evolutionary relationship, phylogenetic tree of each isolated strain was built up using the selected database sequences through the blast search along with the query sequence. Phylogenetic trees were constructed using MEGA-X software following the statistical method of maximum likelihood including 1000 bootstrap replications.

## **Results and Discussion**

### ***Identification of the isolated fungi***

A total 5 endophytes were isolated from the rhizome (bark) (ZOBE-1, ZOBE-2), petiole (ZOPE-3) and leaf parts (ZOLE-1, ZOLE-2) of *Zingiber officinale* (Fig. 1). All the isolated endophytes were morphologically identified up to the genus level and up to the species level through molecular identification.

### ***Morphological identification***

According to the morphological characteristics, four endophytes belongs to *Fusarium* sp. (ZOBE-1, ZOBE-2, ZOPE-3 and ZOLE-1); and another one belongs to *Cladosporium* sp. (ZOLE-2). Identification was based on describing the colony characteristics of 12 days cultural growth according to macroscopic and microscopic point of views explained in Tables 1,2 and 3 which were also verified as confirmed by the previously described features.

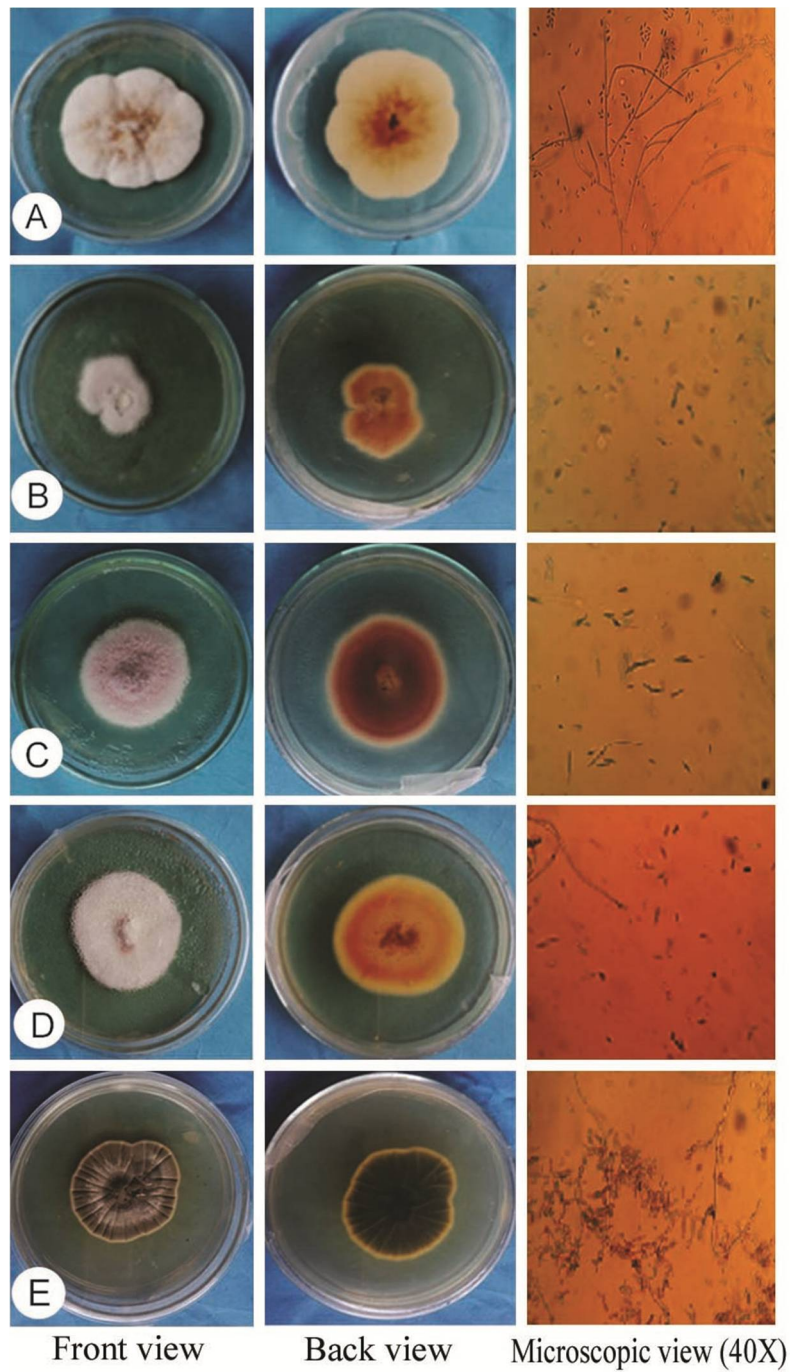


Fig. 1. Isolated endophytic fungi from *Zingiber officinale*. (A) ZOBE-1 (*Fusarium* sp.), (B) ZOBE-2 (*Fusarium* sp.), (C) ZOPE-3 (*Fusarium* sp.), (D) ZOLE-1 (*Fusarium* sp.) and (E) ZOLE-2 (*Cladosporium* sp.)

**Table 1. Morphology of the fungal strains isolated from Rhizome (bark).**

Strains	Morphological characterization	Identified genus
ZOBE-1	<p><b>Macroscopic View</b> Upper view of the colony: Brown color observed in center with white side; Lower view of the colony: light yellowish-brown; growth rate: moderate. Hyphae: soft and aerial mycelium; Morphology of colony: villous, irregular and raised elevation.</p> <hr/> <p><b>Microscopic View</b> Mycelium: mass branched; Spores: single celled, small and large, Microconidia: hyaline, delicate, slightly sickle-shaped or almost straight, Macroconidia: sickle-shaped, one to three septa.</p>	<i>Fusarium</i> sp. (Ignjatov <i>et al.</i> , 2019)
ZOBE-2	<p><b>Macroscopic View</b> Upper view of the colony: White color; Lower view of the colony: Light purple; Growth rate: moderate; Hyphae: fertile, growing vertically; Morphology of colony: villous, circular including entire margin.</p> <hr/> <p><b>Microscopic View</b> Mycelium: Branched and thread shaped; Spores: rod shaped, septed; Conidia: slender sickle-shaped, one to three septa</p>	<i>Fusarium</i> sp. (Ignjatov <i>et al.</i> , 2019)

**Table 2. Morphology of the fungal strains isolated from petiole.**

Strains	Morphological characterization	Identified genus
ZOPE-3	<p><b>Macroscopic View</b> Upper view of the colony: Purple and white; Lower view of the colony: deep purple in center with white side; Growth rate: fast; Hyphae: septate; Morphology of colony: cottony, irregular.</p> <hr/> <p><b>Microscopic View</b> Mycelium: Aerial, branched and unbranched; Spores: single and two celled, cylindrical; Microconidia: small, oval, one or two celled; Macroconidia: typically curved like a sickle, three to five septa expanded in the middle of their length.</p>	<i>Fusarium</i> sp. (Ignjatov <i>et al.</i> , 2019; Zainudin <i>et al.</i> , 2017)

**Table 3. Morphology of the fungal strains isolated from leaf.**

Strains	Morphological characterization	Identified genus
ZOLE-1	<p><b>Macroscopic View</b> Upper view of the colony: White; Lower view of the colony: Light yellow; growth rate: rapid. Hyphae: soft and cottony mycelium; Morphology of colony: circular including entire margin..</p> <hr/> <p><b>Microscopic View</b> Mycelium: Aerial; Spores: single and two celled, small and large, Microconidia: Oval shaped, Macroconidia: curved.</p>	<i>Fusarium</i> sp. (Chehri, <i>et al.</i> , 2015)
ZOLE-2	<p><b>Macroscopic View</b> Upper view of the colony: grey-olivaceous; Lower view of the colony: Light yellowish olive green; Growth rate: slow; Morphology of colony: velvety, irregular.</p> <hr/> <p><b>Microscopic View</b> Mycelium: aerial, sparse, diffuse, or sometimes abundantly formed; Spores: rod shaped, septed; Conidia: subglobose, obovoid, ovoid to limoniform, aseptate; Conidiophores: Straight, solitary, unbranched, terminal or lateral and without nodules.</p>	<i>Cladosporium</i> sp. (Torres <i>et al.</i> , 2017)

### Molecular Identification

The 5 fungal isolates had been identified at species level through the molecular identification including ITS gene sequencing, BLASTN (NCBI) database queries and also interpretation of the phylogenetic analysis respectively. Table 4 explained the list of those best matched organisms that are obtained after BLASTN programs search of the respective strain sequences.

**Table 4. Blast results outputs of the selected isolated strains.**

SL	Strains	Query cover	Percent Identity	Organisms with highest similarity including Accession No.
1.	ZOBE-1	100%	100%	<i>Fusarium proliferatum</i> (HF930594.1)
2.	ZOBE-2	100%	100%	<i>Fusarium proliferatum</i> (LS422790.1)
3.	ZOPE-3	98%	100%	<i>Fusarium proliferatum</i> (MT280199.1)
4.	ZOLE-1	100%	99.64%	<i>Fusarium solani</i> (KT184398.1)
5.	ZOLE-2	100%	100%	<i>Cladosporium cladosporioides</i> (MG572365.1)

### Phylogenetic analysis

Phylogenetic analysis is a method to elucidate the evolutionary history and relationship among a group of organisms. A phylogenetic tree is a diagram that represents evolutionary relationships among organisms. In a phylogenetic tree, the relatedness of two species has a very specific meaning. Two species are more related if they have a more recent common ancestor, and less related if they have a less recent common ancestor. The species or groups of interest are found at the tips of lines referred to as the tree's branches. The pattern of branching in a phylogenetic tree reflects how species or other groups evolved from a series of common ancestors. Each branch point (also called an internal node) represents a divergence event, or splitting apart of a single group into two descendant groups known as a clade. The branch lengths estimate the genetic distance, whereas the branch values represent the bootstrap confidence values.

Regarding the phylogram of Fig. 2 in which ZOBE-1 falls outside the polyphyletic group of diverse species, is located just adjacent to *Fusarium proliferatum* (Acc. no. HF 930594.1) that are strongly supported due to the highest bootstrap value of 82%. Morphological verifications and also such distant relationship distinguishing ZOBE-1 as *Fusarium proliferatum* (Ignjatov, *et.al.*, 2019).

In Fig. 3, ZOBE-2 falls outside the polyphyletic group of diverse species, is located just adjacent to *Fusarium proliferatum* (Accession no. LS 422790.1) that are weakly supported due to low bootstrap value which may be caused by the poor alignment. However, according to the exploration of BLASTN (NCBI) database queries where most of the search results obtained for the same respective organism and also the previous morphological investigations, ZOBE-2 can be confirmed as *Fusarium proliferatum* (Zainudin *et al.*, 2017).

In Fig. 4, ZOPE-3 falls outside the polyphyletic group of diverse species, is located just adjacent to *Fusarium proliferatum* (Accession no. MT 280199.1) that are weakly supported due to low bootstrap value which may be caused by the poor alignment. However, according to the exploration of BLASTN (NCBI) database queries where most of the search result obtained for the same respective organism and also the previous morphological investigations, ZOBE-2 can be confirmed as *Fusarium proliferatum* (Zainudin *et al.*, 2017).

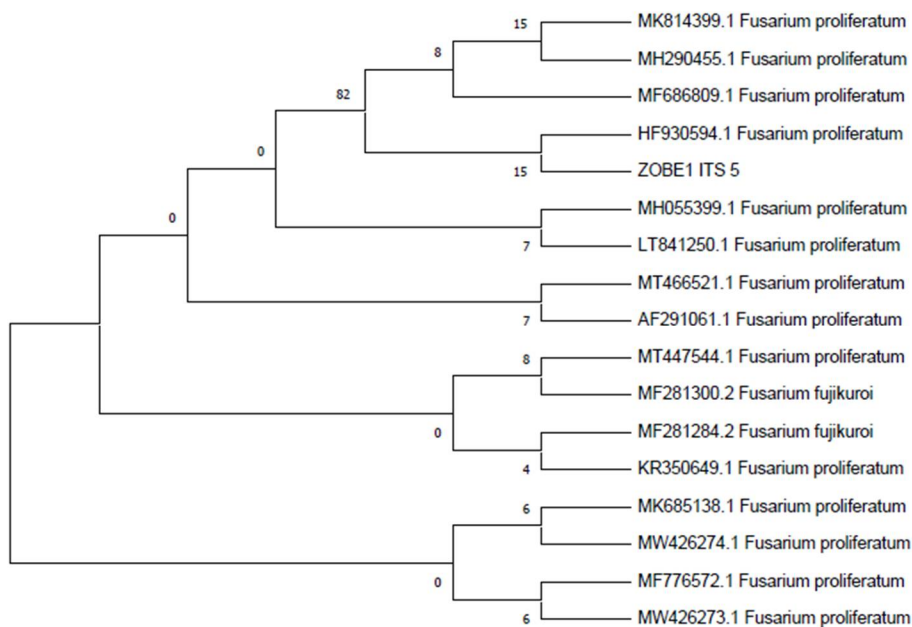


Fig. 2. Phylogenetic relationship between Zobe-1 and the other related species constructed using maximum likelihood method (1000 bootstrap replication) including the bootstrap values supported each node.

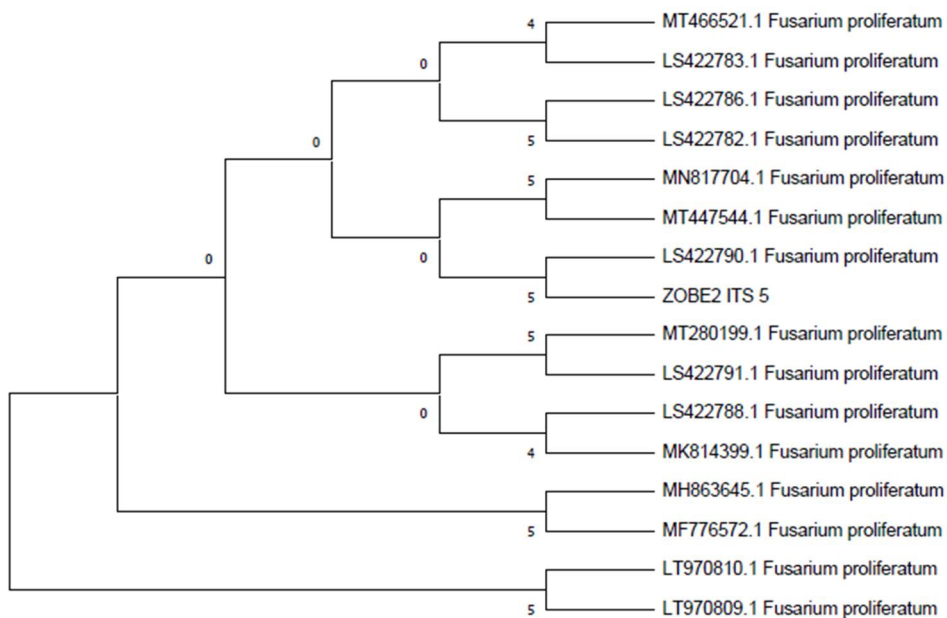


Fig. 3. Phylogenetic relationship between Zobe-2 and the other related species constructed using maximum likelihood method (1000 bootstrap replication) including the bootstrap values supported each node.

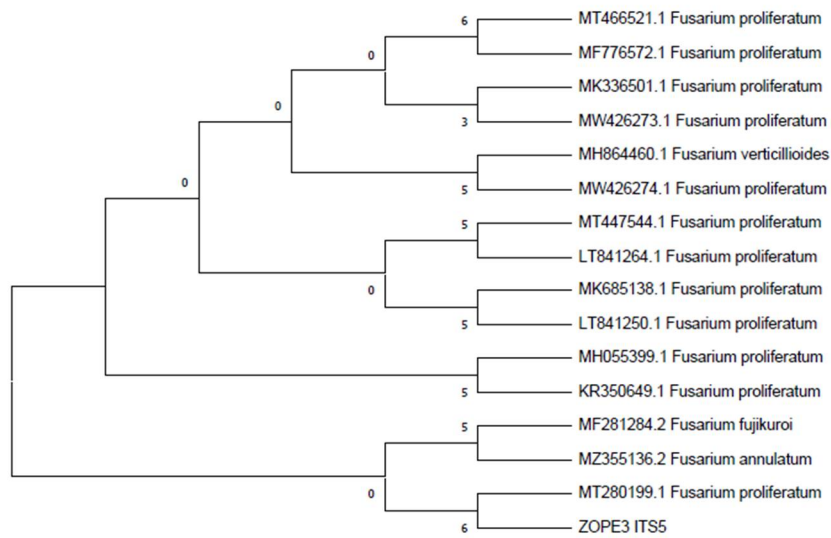


Fig. 4. Phylogenetic relationship between ZOPE-3 and the other related species constructed using maximum likelihood method (1000 bootstrap replication) including the bootstrap values supported each node.

Regarding the phylogram of Fig. 5 in which ZOLE-1 falls outside the polyphyletic group of diverse species that are strongly supported due to bootstrap value of 36% (Accession no. KT184398.1). Morphological verifications and also such distant relationship distinguishing ZOLE-1 as *Fusarium solani* (Chehri *et al.*, 2015).

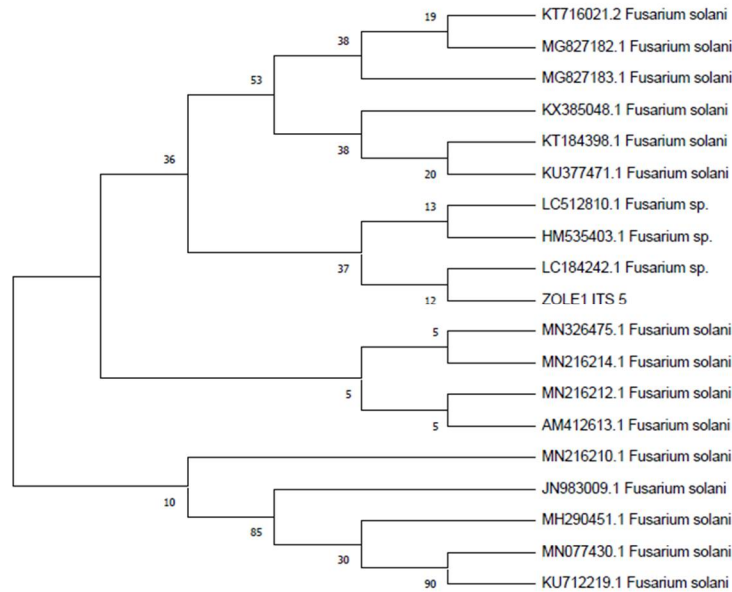


Fig. 5. Phylogenetic relationship between ZOLE-1 and the other related species constructed using maximum likelihood method (1000 bootstrap replication) including the bootstrap values supported each node.



In case of Fig. 6, where ZOLE-2 serves as an outgroup positioning beyond the paraphyletic clade of several *Cladosporium* sp., is more distantly related to *Cladosporium cladosporioides* (Accession no. MG 572365.1) with a probably significant bootstrap value of 61%. Following that arrangement, the ZOLE-2 strain can be registered as *Cladosporium cladosporioides* the morphology of which can also viewed as same as the respective strain (Torres *et al.*, 2017).

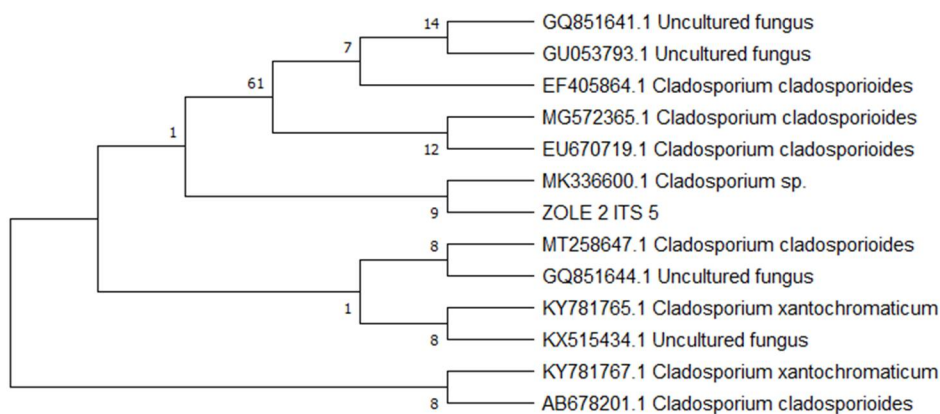


Fig. 6. Phylogenetic relationship between ZOLE-2 and the other related species constructed using maximum likelihood method (1000 bootstrap replication) including the bootstrap values supported each node.

## Discussion

This study was conducted to characterize both morphological and molecular examination following phylogenetic analysis for targeting the proper identification of all the fungi isolated from the plant *Zingiber officinale*. The isolated five strains belong to two genera like *Fusarium* and *Cladosporium*. However, morphological analysis must be compared with the result of molecular examination, as some characteristics are identical between species. For this reason the recognized genera of the isolated fungi were further identified at the species level based on 5.8S-rRNA-ITS sequences. In finding of evolutionary relationship, phylogenetic trees are commonly constructed. A phylogenetic tree sorts organisms into clades or groups of organisms that descended from a single ancestor using maximum parsimony. All the developed phylogenetic trees of the respective strain were found to be reliable based on bootstrap values except for ZOBE-2 and ZOPE-3 because of forming a weak monophyletic clade.

The genera *Fusarium* sp. contains over 300 species and widely distributed in various habitats, aquatic, soil, and plant associated. From the literature it is suggested that they produce many famous bioactive compounds such as equisetin (anti-HIV and anti-bacterial activities) (Jeong and Moloney, 2015), conioisetin (anti-bacterial activity) (Segeth *et al.*, 2003) and fusarisetin A (inhibiting the tumor metastasis) (Jang *et al.*, 2011), and so on. In light of this, the *Fusarium* genus fungi have become a rich and hot source for discovering drug leads. The genus *Cladosporium* includes more than 30 species, with *C. cladosporioides* as one of the most common species. *Cladosporium* sp. was reported to produce several secondary metabolites, including cladospurin, emodin, phytase, taxol, and other antibiotic and antifouling compounds (Quan *et al.*, 2004; Zhang *et al.*, 2009; Xiong *et al.*, 2009). Another study isolated and identified beneficial secondary metabolites (brefeldin A) from an isolated active strain I(R)9-2, *Cladosporium* sp. (Wang *et al.*, 2007). Two naphthoquinones, namely anhydrofusarubin and methyl ether of fusarubin were

isolated from *Cladosporium* sp. by Md. Imdadul Huque Khana *et al.* The isolated compounds showed potential cytotoxicity and prominent antibacterial properties (Khan *et al.*, 2016). Following such previous investigating report, it can be proposed that these identified endophytes can be the great resources of novel antimicrobial or anticancer compounds. The present study provides a basis for such further studies. Secondary metabolites of *Fusarium* sp. and *Cladosporium* sp. can be the preference of our future research.

### Conclusion

The current study defines the structural detection of all the fungal isolates that is confirmed throughout the genetic analysis including their commencing from phylogenetic theory. Such features of these potential fungi can be considered as the most suitable information in research database.

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