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Molecular identification and antimicrobial activity of endophytic fungi isolated from *Heritiera fomes* (Buch. -Ham), a mangrove plant of the Sundarbans

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Abstract

Background: Endophytic fungi play a vital role in plant defense system by secreting various antimicrobial agents. To evaluate the antimicrobial activity of the endophytic fungi of the mangrove plant *Heritiera fomes* (Buch. -Ham), plants were collected from the Sundarbans, Bangladesh. The endophytic fungi were subsequently isolated and identified.

Results: Fifty-five endophytic fungi were isolated from the leaves, root, and bark of *H. fomes* (Buch. -Ham). Genomic DNA was extracted for PCR (polymerase chain reaction) by specific primers to multiply ITS region and sequences were determined. Nucleotide sequence data were submitted to the Genbank and accession number for each fungal strain was obtained. Antimicrobial activity of the ethyl acetate (EtOAc) and methanolic extracts of eleven species from both fermentation and mycelium, respectively, were analyzed by microtiter plate-based antimicrobial assay incorporating resazurin as an indicator of cell growth against two Gram-positive bacteria namely *Staphylococcus aureus* NCTC 12981 and *Micrococcus luteus* NCTC 7508, two Gram-negative bacteria namely *Escherichia coli* NCTC 12241 and *Pseudomonas aeruginosa* NCTC 12903, and a fungus *Candida albicans* ATCC 90028. All the endophytic fungal extracts exhibited antimicrobial activities against more than one-tested pathogenic microbial strains. Overall, methanolic extracts showed greater activity than EtOAc extracts. *Pseudopestalotiopsis camelliae-sinensis*, *Pestalotiopsis microspora*, and *Penicillium copticola* were the most active endophytic fungal strains and exhibited strong inhibitory activity against the microorganisms under investigation and their MIC values ranged from 0.0024 to 5.0 mg/mL. Methanolic extracts of both *P. camelliae-sinensis* and *P. microspora* showed the highest antibacterial activity (MIC value of 0.0024 mg/mL) against *P. aeruginosa* NCTC 12903.

Conclusion: This study showed that the isolated and identified endophytic fungi from *H. fomes* (Buch. -Ham) could be potential sources of antimicrobial agents.

Keywords: Antimicrobial, Endophytic fungus, Mangrove plants, *Heritiera fomes*, The Sundarbans

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1 Background

In recent times, the chemistry of endophytic fungi has drawn considerable amount of interest as promising sources of new antibiotics and other therapeutic agents to face the emerging challenges in the arena of medicine and environment [1]. Many endophytic fungi have been reported to exhibit a number of interesting potential bioactivities through synthesis of diverse secondary metabolites by different metabolic pathways [2–4], viz. polyketides, isoprenoids, or derivatives of amino acids like phenols, steroids, xanthenes, perylene derivatives, isocoumarins, quinines, furandiones, terpenoids, depsi-peptides, and cytochalasins [5, 6]. Endophytes protect their hosts against various biotic and abiotic stresses by producing various bioactive compounds and provide unique mechanisms to support survival of the host plants [6]. Endophytic fungi reside in the intercellular spaces without showing any symptoms and do not cause adverse effects on their hosts [7]. Moreover, plants gain protection against invaded pathogenic microbes and herbivores because of the biosynthesis of bioactive secondary compounds by endophytic fungi [8]. Plant extracts have provided health benefits and long been used as main component of traditional medicines to fight against diseases and ailments [9]. However, many medicinal plants have been reduced from the natural habitat by over utilization, environmental deterioration, climate change, and ineffective conservation approaches. Several studies have shown that endophytic fungi can synthesis bioactive secondary compounds, analogous to the plant secondary metabolites [10]. Thus, endophytic fungi could be an alternative and untapped resource of potential bioactive compounds from the wild rather utilization of plant resources and their mass exploitation. They could be subjected to mass cultivation and further amenable to microbial fermentation techniques.

It has been well established that ecology of plants has enormous effect on the genetic diversity of the endophytes obtained from the plants and this has enabled them to produce different types of metabolites [11]. It is noteworthy to mention that a very few studies have been conducted to explore the diversity of mangrove endophytic fungi from the Sundarbans, the largest mangrove forest in the world [12–15]. We hypothesize that the endophytic fungi isolated from mangrove plants thriving at highly saline environment would produce a myriad of bioactive compounds to provide protection against salinity-induced abiotic-stress [16, 17], and these compounds may possess bioactive properties. Considering these facts, *Heritiera fomes* (Buch. -Ham), a primary mangrove plant and one of the dominant species of the world's largest mangrove forest the Sundarbans, has been chosen in the present study. In addition, this plant has never been studied for the presence of bioactive

endophytic fungi and their activities. It is an evergreen moderate size tree growing abundantly in Sundarbans [18] and the trees can grow up to 25 m in height. The shining golden-brown scales are seen on the young branches of the trees. *H. fomes* is regarded as one of the important mangrove species having ethnomedicinal uses in traditional medicines. The people living in the vicinity of the mangrove forest use the plant extensively for treatment of various ailments. Common uses include treatments for gastrointestinal disorders including diarrhea, dysentery, constipation, indigestion, and stomach-ache. In addition, the plant is recommended for treatment of skin diseases including dermatitis, rash, eczema, boils, itch, scabies, sores, infections, and hepatic disorders including jaundice, hepatitis. Sometimes, traditional health practitioners use the plant for treating diabetes and goiter. It is a good insect repellent [19] and has wound healing activity [20]. *H. fomes* possesses significant antioxidant [21], antinociceptive, antihyperglycemic [22], antimicrobial [23], and anticancer activities [24]. It is also useful in cardiovascular diseases [25]. Though most of the findings have focused on these several bioactivities, endophytic fungi of the plant were never identified and characterized.

Therefore, the present study was carried out to isolate and identify endophytic fungi associated with mangrove plant *H. fomes* (Buch. -Ham) of the Sundarbans and subsequently subjected to evaluation of antimicrobial activity of the isolated fungal extracts against several pathogenic microorganisms.

2 Methods

2.1 Collection of plant sample, preparation, and isolation of endophytic fungi

Healthy and young roots, bark, and leaves of *H. fomes* (without any sign and symptoms of infection) were collected from the mangrove forest, the Sundarbans, Khulna, Bangladesh. The plant *H. fomes* was identified during sample collection based on the morphological characteristics described by Zabala [26] and a voucher specimen (TRN-KU-2017011) of this collection was retained. Plant samples were kept in sterile polythene bags under humid conditions at room temperature. Isolation of endophytic fungi was carried out by the procedure as described by Nurunnabi et al. [12]. Briefly, plant materials were washed thoroughly in sterile water to eliminate extraneous substances. The samples were then surface sterilized by sequentially immersing them in 70% ethanol for 30 s and 5% sodium hypochlorite solution for 1 min and finally rinsed with sterile distilled water. The samples were then surface-dried with sterile filter paper. Roots, bark, and leaves were cut into 0.5 cm × 0.5 cm pieces and placed on aqueous agar media (2% agar-agar in distilled water) supplemented with antibiotic

streptomycin (30 mg/L) and incubated at 28 ± 2 °C until fungal growth was initiated. The tips of the fungal hyphae were removed from the aqueous agar media and placed on potato dextrose agar (PDA) media. After 7 days of incubation, colony morphology was assessed to determine the purity of each fungal culture. The isolates were cultured several times on PDA to obtain final pure cultures and then transferred to PDA slants for storage until further study.

2.2 Macroscopic, microscopic, and molecular identification of the fungal isolate

Macroscopic, microscopic, and molecular techniques were employed to identify fungal endophytes according to the method described by Qadri et al. [27]. Macroscopically, the fungal morphology was studied by observing the colony features (shape, size, color, and hyphae) and microscopically, slides were prepared by mounting a small portion of the mycelium and then stained with lactophenol cotton blue. The slides were then examined by a compound microscope with a digital camera [28]. For molecular identification, fungal DNA was isolated using fungal DNA isolation kit (Cat-26200; NORGEN BIOTEK Corp., 3430 Schmon Parkway, Thorold, ON, Canada). Phylogenetic analysis of the isolated fungi was carried out by sequencing internal transcribed spacer (ITS) region of ribosomal gene. The ITS region of the fungi was amplified using the forward primer ITS4 (5' TCCTCC GCTTATTGATATGC3') and reverse primer ITS5 (5' GGAAGTAAAAGTCGTAACAAGG 3'), using polymerase chain reaction (PCR) [27].

The PCR reaction was performed in 50 μ L reaction volume comprising 10 μ L of 5X reaction buffer, 3 μ L of 1.5 M $MgCl_2$, 5 μ L of 10 mM dNTP, 3 μ L of 100 pmol primers, 26 μ L of distilled water, and 3 μ L (2 ng/ μ L) of DNA. The PCR amplification was performed using 2720 PCR Thermo Cycler and the temperature profile was maintained as initial denaturation at 94 °C for 5 min followed by 39 cycles for each denaturation (94 °C for 30 s), annealing of the primers (51 °C for 30 s), and extension (72 °C for 2 min). The final extension was carried out at 72 °C for 5 min and held at 4 °C. Then, amplified products (10 μ L) were verified by electrophoresis using 1.5% agarose gel for 45 min at 400 mA current flow. After carefully staining the gel with ethidium bromide, the amplified DNA fragments were observed under UV light transilluminator. The rest of the PCR amplified products (40 μ L each) were purified according to requirement of Cambridge Genomic Services (CGS) (The University of Cambridge, UK) for sequencing. Consensus sequences (~522–560 bp) of 5.8S rRNA were generated from forward and reverse sequence data using aligner software MEGA 7.0. Multiple BLASTN searches were made against the sequence available in the National

Center for Biotechnology Information (NCBI). Consensus sequences (5.8S rRNA) of isolated fungi were submitted in the GenBank of NCBI for acquisition of accession numbers. Unweighted pair group method with arithmetic mean (UPGMA) tree was constructed and pair wise genetic distance was obtained by the PAUP 4.0 software. Phylogenetic tree was constructed to show the genetic similarity among the isolated fungi. Briefly, the tree was constructed based on rDNA sequence (ITS1, 5.8S, and ITS 4) by using neighbor-joining method. The bootstrap consensus tree inferred from 1000 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were 522 positions in total in the final dataset. Phylogenetic analyses were conducted by PAUP 4.0 software.

2.3 Preparation of endophytic fungal extracts

The fungal isolates were cultured into (5 \times 250 mL) conical flasks containing potato dextrose broth (PDB) for 28 days at 28 °C with 180 rpm of continuous shaking. Fungal mycelia were separated through filtration (Whatman® qualitative filter paper, Grade 1; Sigma-Aldrich, USA) and the filtrates were extracted three times with an equal volume of ethyl acetate (EtOAc) using a separating funnel. The mycelium was grounded and dissolved in methanol for 2 days at dark and the mycelium was again separated by filtration. The EtOAc and the methanolic extracts were evaporated under reduced pressure at 40–45 °C and 45–50 °C, respectively, using rotary evaporator to obtain crude EtOAc and methanolic extracts [29].

2.4 Antimicrobial screening

Both EtOAc and the methanolic extracts of eleven isolated fungi were tested for their potential antimicrobial activities using the resazurin 96-well microtiter plate-based in vitro antimicrobial assay described by Sarker et al. [30]. The antimicrobial activities were tested against two Gram-positive bacterial strains viz. *Staphylococcus aureus* (NCTC 12981) and *Micrococcus luteus* (NCTC 7508), and two Gram-negative viz. *Escherichia coli* (NCTC 12241) and *Pseudomonas aeruginosa* (NCTC 12903) as well as a fungal strain, *Candida albicans* (ATCC 90028). Ciprofloxacin and Nystatin were used as a positive control for bacterial and fungal strain, respectively. Microtiter plates were labeled and then A volume of 100 μ L of crude extract (10 mg/mL) in 10% (v/v) DMSO was pipetted into the first row of the plate and remainder wells of the plate were filled aseptically with 50 μ L normal saline. Serial dilution technique was performed in such a way that each well contained 50 μ L of the test material in descending concentrations. To each

well, 30 μ L of nutrient broth and 10 μ L of resazurin (0.2 mg/mL) were added as an indicator of cell growth. Finally, 10 μ L of microbial suspension (5×10^5 cfu/mL) was added to each well and plates were loosely wrapped with cling film. Each plate had a set of controls: a column with the antibiotic as positive control as well as a column with 10 μ L of nutrient broth along with all solutions instead of 10 μ L microbial solution. The plates were replicated three times and incubated at 37 °C for 18–24 h for any color change. Color change to pink or colorless indicated the positive microbial activity. The lowest concentration of the crude extract at which the change of color took place was considered the MIC value.

2.5 Calculation and statistical analysis

Colonization rate (CR) and isolation rate (IR) were calculated as percentages. For CR, total number of plant tissue segments infected by fungi divided by the total number of segments incubated, was used to indicate comparison of degrees of different tissues infected by endophytic fungi. IR was used to express the fungal richness in a given sample of plant tissue which was calculated as the numbers of isolates obtained from plant segments divided by the total number of segments incubated. The statistical analyses were done by Graph Pad Prism, version 6.01.

3 Results

3.1 Isolation and identification of endophytic fungi

A total of 55 endophytic fungi were isolated from 90 tissue segments (30 segments of each for roots, bark, and leaves) of *H. fomes* (Buch. -Ham). The isolation frequency of roots, bark and leaves were 50, 60, and 73% [$F(2, 6) = 54.41, P < 0.05$], respectively. The isolation frequency of endophytic fungi from leaves was significantly higher than that of bark and roots. The colonization rate

of endophytic fungal isolates varied significantly from each other [$F(7, 16) = 193.09, P < 0.05$] and it was found that *Alternaria spp.* showed highest colonization rate (25.6%) while *Nigrospora spp.*, *Penicillium spp.*, and *Pestalotia spp.* showed 10.0%, 8.9%, and 7.8% colonization rates, respectively. The colonization rates for *Epicoccum spp.*, *Cladosporium spp.*, *Bjerkandera spp.*, and *Lasiodiplodia spp.* were lower than the abovementioned isolates with the values of 3.3%, 2.2%, 2.2%, and 1.1%, respectively. After isolation of endophytes, isolates were grouped into eleven types based on their macroscopic and microscopic characteristics. Among the eleven isolated endophytic fungi from *H. fomes* (Buch. -Ham), light micrographs of two isolates are presented in Fig. 1. One from each group out of eleven groups was subjected to genomic DNA isolation. ITS region of the fungi was multiplied by ITS4 and ITS5 universal primers. Approximately 550 bp of ITS region was generated by PCR reaction. ITS region amplified from genomic DNA of 11 endophytic fungi is shown in Fig. 2. PCR products were sent to Cambridge Genomic Services (CGS), the University of Cambridge, UK, for sequencing. By sequence determination and comparison with the sequences of Genbank database, the strains were identified to the species level (97 to 100% homology to the best-matching reference sequence). Obtained DNA sequences were submitted to the Genbank and accession numbers for each fungal isolate were obtained. Fifty-five endophytic fungi belonging eleven taxa were identified. Table 1 represents the isolated endophytic fungi from *H. fomes* (Buch. -Ham) with NCBI accession numbers. We found maximum similarity (100%) in the isolates for *P. copticola*, *A. alternata*, and *L. theobromae*, respectively, with the sequences of reference strains deposited in Genbank in the BLAST match sequence comparison. However, *L. theobromae* and *A. alternata* were found to be the frequent genera obtained from *H. fomes*.

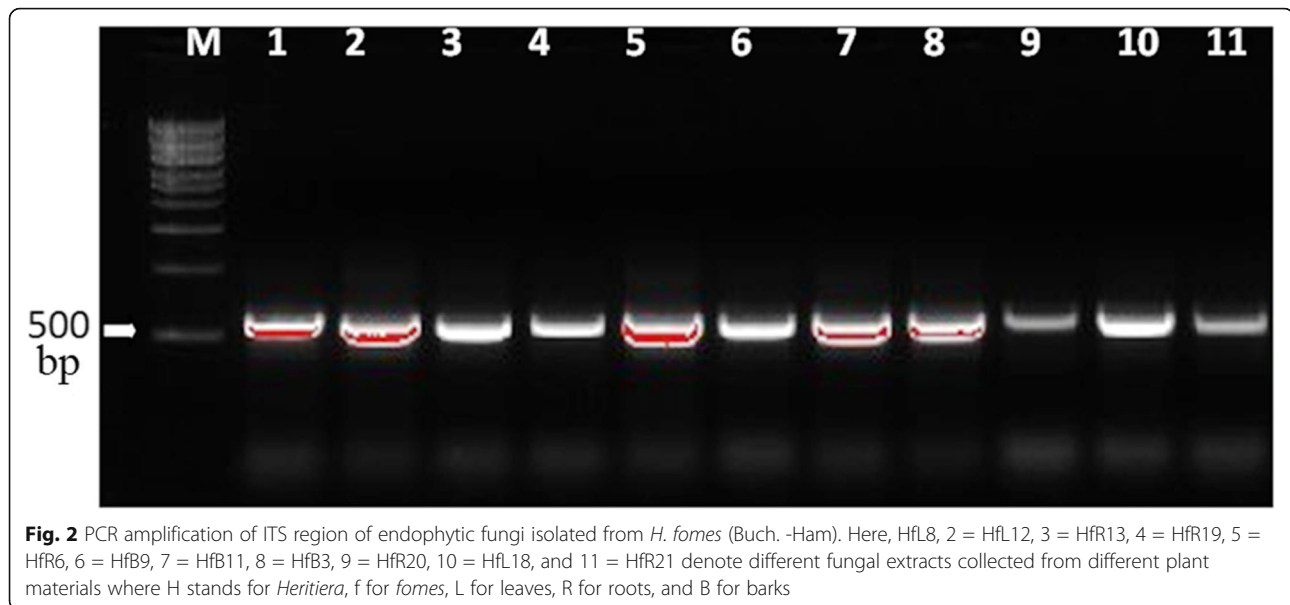


a



b

Fig. 1 Representative light micrographs of two endophytic fungi isolated from *H. fomes* (Buch. -Ham) on PDA media. **a** HfR14 (*Neopestalotiopsis microspora*). **b** HfR19 (*Alternaria alternata*)



3.2 Phylogenetic tree and pair wise genetic distance

The phylogenetic tree constructed by PAUP 4.0 software showed two main branches (Fig. 3). It was represented that HfL8-MH220352 (*L. theobromae*), HfR20-MH237826 (*B. adusta*), HfR21-MH220288 (*E. nigrum*), HfL18-MH220349 (*C. tenuissimum*), and HfR19-MK757987 (*A. alternata*) had clustered in one group while rest of the fungi belonged to another group. HfB9-MH211227 (*N. sphaerica*) and HfB11-MH211241 (*N. oryzae*), HfR20-MH237826 (*B. adusta*), and HfR21-MH220288 (*E. nigrum*) as well as HfL18-MH220349 (*C. tenuissimum*) and HfR19-MK757987 (*A. alternata*) have minimum genetic distance so they are close together.

Pair wise genetic distance (Table 2) expressed genetic distance among the endophytic fungi from *H. fomes* (Buch. -Ham). Maximum genetic distance (0.789) was

found between HfL19 (*A. alternata*) and HfR20 (*B. adusta*). In contrast, minimum genetic distance was found between HfB9 (*N. sphaerica*) and HfB11 (*N. oryzae*).

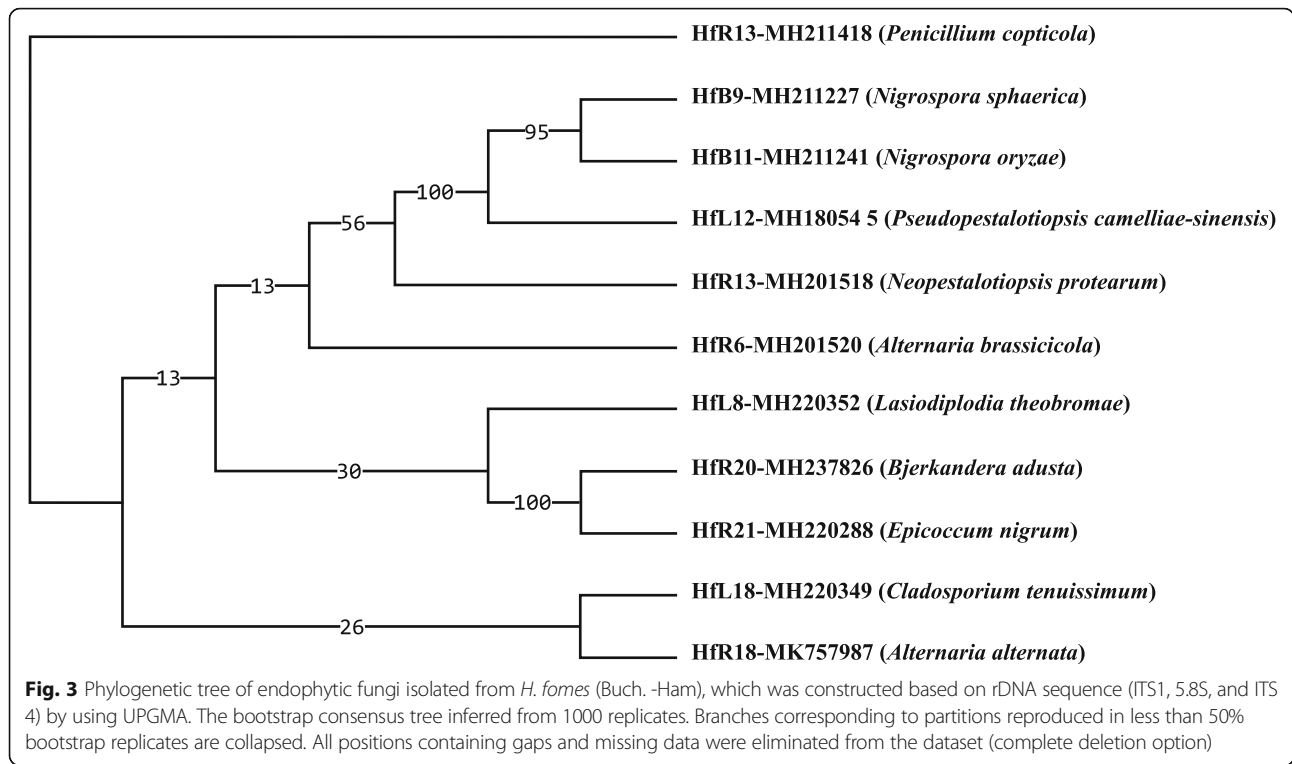
3.3 Antimicrobial activity of the methanolic and ethyl acetate extract of the endophytic fungi

All the crude EtOAc and methanolic extracts of the isolates showed inhibitory effects against more than one tested organism (Table 3). Ciprofloxacin and Nystatin were used as positive control for bacteria and fungus, respectively. On average, the methanolic extracts were more effective on the tested microorganisms. It was found that about 97% (48/55) of methanolic extracts showed better antimicrobial activities than 63% (35/55) of ethyl acetate extracts. Methanolic and EtOAc extracts from *N. microspora*, *P. camelliae-sinensis*, and *P.*

Table 1 Endophytic fungi isolated from *H. fomes* (Buch. -Ham) with NCBI accession numbers

Internal code	Name of the fungi	Genbank accession number	BLAST match sequence		
			Reference accession no.	Query length	Max iden.
HfL12	<i>Pseudopezalotiopsis camelliae-sinensis</i>	MH180545	<i>Pseudopezalotiopsis camelliae-sinensis</i> KX757714.1	536	99%
HfR14	<i>Pestalotiopsis microspora</i>	MK778440	<i>Pestalotiopsis microspora</i> MH712257.1	528	98%
HfR19	<i>Alternaria alternata</i>	MK757987	<i>Alternaria alternata</i> KT898758.1	539	100%
HfR6	<i>Alternaria brassicicola</i>	MH203320	<i>Alternaria brassicicola</i> KY310727.1	559	97%
HfB9	<i>Nigrospora sphaerica</i>	MH211227	<i>Nigrospora sphaerica</i> KX256179.1	534	98%
HfB11	<i>Nigrospora oryzae</i>	MH211241	<i>Nigrospora oryzae</i>	530	99%
HfB3	<i>Penicillium copticola</i>	MH211418	<i>Penicillium copticola</i> NR_121516.1	556	100%
HfR20	<i>Bjerkandera adusta</i>	MH237826	<i>Bjerkandera adusta</i> MF120203.1	560	98%
HfL18	<i>Cladosporium tenuissimum</i>	MH220349	<i>Cladosporium tenuissimum</i> MF473305.1	532	99%
HfR21	<i>Epicoccum nigrum</i>	MH220288	<i>Epicoccum nigrum</i> JF440590.1	525	99%
HfL8	<i>Lasiodiplodia theobromae</i>	MH220352	<i>Lasiodiplodia theobromae</i> JX868719.1	522	100%

H, *Heritiera*; f, *fomes*; L, leaves; R, root; B, bark



copticola showed antimicrobial effect on all tested microorganisms and only the methanolic extract from *N. sphaerica* and *N. oryzae* showed activity against all of them. The methanolic extract from *N. microspora* and *P. camelliae-simithae* confirmed the highest activity with an MIC of 0.0024 mg/mL against *P. aeruginosa* (NCTC 12903). The fungal extract displayed activity against *P. aeruginosa* (NCTC 12903) and *S. aureus* (NCTC 12981) (MIC ranged from 10 to 0.00024 mg/mL). Only 40% (9/22) and 59% (13/22) of the extracts showed activity against *E. coli* (NCTC 12241) and *C. albicans* (ATCC

90028), respectively. For *M. luteus* (NCTC 7508), 81% (18/22) of the extracts exhibited antibacterial activity. EtOAc and methanolic extracts from *P. copticola* showed significant effect against *M. luteus* (NCTC 7508) with MIC value of 0.0097 mg/mL in both cases.

4 Discussion

Endophytic fungi have been reported from a variety of plant species that contribute to the diversity of microorganisms in the ecosystems. Inside the plant, the fungi produce various biologically active compounds which

Table 2 Pair wise genetic distance of eleven endophytic fungi isolated from *H. fomes* (Buch. -Ham)

	HfB3	HfB9	HfB11	HfL8	HfL12	HfL18	HfR6	HfR13	HfR19	HfR20	HfR21
HfB3	-										
HfB9	0.695	-									
HfB11	0.693	0.317	-								
HfL8	0.733	0.745	0.731	-							
HfL12	0.739	0.411	0.419	0.747	-						
HfL18	0.699	0.716	0.701	0.741	0.733	-					
HfR6	0.747	0.766	0.779	0.745	0.758	0.766	-				
HfR13	0.704	0.714	0.712	0.772	0.628	0.724	0.722	-			
HfR19	0.716	0.747	0.764	0.760	0.749	0.722	0.731	0.766	-		
HfR20	0.758	0.731	0.756	0.762	0.762	0.789	0.758	0.764	0.758	-	
HfR21	0.747	0.735	0.722	0.743	0.756	0.770	0.760	0.783	0.752	0.429	-

H, *Heritiera*; f, *fomes*; L, leaves; R, root; B bark

Table 3 Minimum inhibitory concentration (MIC, in mg/mL) of ethyl acetate (EtOAc) and methanolic (MeOH) extracts of 11 different endophytic fungi from *H. fomes* (Buch. -Ham)

Name of the endophytic fungi	Extract	Gram-negative bacteria		Gram-positive bacteria		Fungal Strain
		<i>E. coli</i> (NCTC 12241)	<i>P. aeruginosa</i> (NCTC 12903)	<i>M. luteus</i> (NCTC 7508)	<i>S. aureus</i> (NCTC 12981)	<i>C. albicans</i> (ATCC 90028)
<i>Pseudopestalotiopsis camelliae-sinensis</i>	EtOAc	0.312	0.312	0.312	0.125	0.625
	MeOH	5	0.0024	0.625	0.312	0.312
<i>Pestalotiopsis microspora</i>	EtOAc	5	0.125	0.25	0.25	0.312
	MeOH	0.25	0.0024	0.625	0.125	0.625
<i>Alternaria alternata</i>	EtOAc	NA	2.5	1.25	10	NA
	MeOH	NA	1.25	0.625	5	2.5
<i>Alternaria brassicicola</i>	EtOAc	NA	5	1.25	10	NA
	MeOH	NA	1.25	1.25	5	10
<i>Nigrospora sphaerica</i>	EtOAc	NA	5	NA	5	NA
	MeOH	10	0.625	1.25	0.625	10
<i>Nigrospora oryzae</i>	EtOAc	NA	5	NA	5	NA
	MeOH	10	0.625	1.25	0.625	5
<i>Penicillium copticola</i>	EtOAc	2.5	0.625	0.0097	0.625	1.25
	MeOH	2.5	0.625	0.0097	0.312	1.25
<i>Bjerkander aadusta</i>	EtOAc	NA	NA	NA	5	10
	MeOH	NA	5	1.25	10	NA
<i>Cladosporium tenuissimum</i>	EtOAc	NA	1.25	2.5	10	NA
	MeOH	5	2.5	2.5	5	NA
<i>Epicoccum nigrum</i>	EtOAc	NA	2.5	10	10	NA
	MeOH	NA	2.5	5	5	2.5
<i>Lasiodiplodia theobromae</i>	EtOAc	NA	0.62	NA	2.5	NA
	MeOH	NA	0.62	5	1.25	2.5
Ciprofloxacin		0.00049	0.00012	0.00098	0.00098	NT
Nystatin		NT	NT	NT	NT	0.00098

NA no activity, NT not tested, EtOAc ethyl acetate extract, MeOH methanolic extract

play versatile roles in their inherent surroundings. Mangroves plants can thrive in a unique habitat with high microbial and fungal competitions and brackish tidal environment. The association with endophytic fungi and mangrove plants confers protection from unsympathetic environmental conditions and allow them to cope with saprobic fungi [31]. In an extensive review, Liu et al. (2007) documented more than 200 species of mangrove endophytic fungi and predominant species included *Alternaria*, *Aspergillus*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Paecilomyces*, *Penicillium*, *Pestalotiopsis*, *Phoma*, *Phomopsis*, *Phyllosticta*, and *Trichoderma* [32]. They also reported that most endophytic fungi could originate from wide range of hosts which a few might have single host [33]. In another study, *Colletotrichum spp.* was found to be one of the most abundant endophytic fungal species in Indian mangrove plant community [34]. In Pichavaram mangrove ecosystem, several endophytic fungal genera have been reported from the

leaves of various mangrove plants [35]. Our study is also in good agreement with these previous studies as we have isolated and identified several distinctive endophytic fungal species in different genera. However, in our case, *Alternaria spp.* was the most dominant species followed by *Nigrospora spp.* and *Penicillium spp.*

Although we identified the endophytic fungus based on morphological characteristics, however, this type of identification is presumptive and requires fair bit of experience. Therefore, in addition to this, we chose nucleotide sequence analysis for more accurate identification, due to its simplicity and straightforward nature of operation. In the present study, ITS region was chosen because of its location between two highly conserved genes, which code for 18S and 28S rRNA. The ITS regions circumscribe two noncoding regions ITS1 and ITS2, which are separated by the highly conserved 5.8S rRNA gene [36]. The ITS1 and ITS2 regions are more variable than the other adjacent rRNA gene sequences

[37] and, therefore, offer a better identification of closely related species. By sequence determination and comparison with the sequences of Genbank database, all the strains were identified to the species level (97 to 100% homology to the best-matching reference sequence).

On average, methanolic extracts were found to be the most effective, except for *P. camelliae-sinensis* and *N. protearum* where the extracts of ethyl acetate were more active than that of methanolic extracts. Ethyl acetate extracts from these two fungi showed better activities against the test microorganisms. In other cases, methanol was found to be suitable and effective as an extraction medium and methanolic extracts showed better antimicrobial activities than ethyl acetate against the tested microbes. The data is supported by the pronounced antimicrobial effects shown by methanolic extracts over ethyl acetate extracts, which is evident from the level of inhibition of growth for various microorganisms under investigation and can be associated with the change of the color for resazurin. The change of the color in the well is inferred that the metabolic activity of live microbes released CO₂ and O₂ which became pink and fluorescent when reduced to resorufin by oxidoreductase activity. Resorufin is further reduced to hydroresazurin and becomes colorless. Due to inhibition of bacterial action by potential fungal extracts, the dye color stays unaffected in the well. From this assay, fungal extracts which possessed antimicrobial potential were identified rapidly [30]. In a study of Brazilian mangrove plant *Laguncularia racemosa* (L.) Gaertn, seventy endophytic fungal strains were isolated from leaves and antimicrobial properties of their ethyl acetate extracts were evaluated against *S. aureus*, *Bacillus subtilis*, *E. faecalis*, *M. luteus*, *E. coli*, and *P. aeruginosa*. The results showed that endophytic fungi strains were able to produce secondary metabolites with antimicrobial activity, and the crude extracts from *Aspergillus niger*, *Curvularia pallescens*, *Guignardia bidwellii*, *Paecilomyces variotii*, and *Mycelia sterilia* showed the best results [38]. In another study, Bhimba et al. (2012) investigated the antibacterial activity of marine-derived fungi *Phoma herbarum*, isolated from mangrove leaves. The isolated fungi showed potent activity against *M. luteus* and *Vibrio cholerae* [39]. Similarly, a mangrove fungi *P. microspora* VB5 was isolated from the leaves of two mangrove plants from Pichavaram mangrove forest in Southeast coast of India [40]. The crude extracts of this endophytic fungi showed strong antimicrobial activity against *B. subtilis* (ATCC 6633), *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), and *P. aeruginosa* (ATCC 27853) by agar well diffusion method.

5 Conclusion

In this study, we successfully isolated and identified 11 different species of endophytic fungi belonging to 9

different genera. All the isolates are moderately active against tested microorganisms. However, further studies could be initiated with *N. microspora*, *P. camelliae-sinensis*, and *P. copticola* for potential for bioactive compounds as our results showed promising antimicrobial activities with these endophytic fungi. The findings of this study also suggested that endophytes from harsh and competitive environments, such as mangrove ecosystem, might be an attractive source for bioprospecting of new anti-infective compounds.

Abbreviations

CGS: Cambridge Genomic Services; CR: Colonization rate; EtOAc: Ethyl acetate; IR: Isolation rate; ITS: Internal transcribed spacer; NCBI: National Center for Biotechnology Information; PCR: Polymerase chain reaction; PDA: Potato dextrose agar; UPGMA: Unweighted pair group method with arithmetic mean

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Authors' contributions

Conceptualization: MMB, SMMR, SDS, and MHS. Methodology: MMB and TRN. Formal analysis and investigation: TRN. Data and statistical analysis: TRN, FS, and FA. Writing—original draft preparation: TRN, FS, SS, and FA. Writing—review and editing: MMB, TRN, FS, SS, and FA. Funding acquisition: MMB, SMMR, SDS, MHS, and LN. Resources: MMB, SMMR, SDS, MHS, and LN. Supervision: MMB, SMMR, SDS, MHS, and LN. All authors have read and approved the manuscript.

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Competing interests

The authors declare that they have no competing interests.

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