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Diversity And Molecular Phylogeny of Fungal Endophytes Isolated from An Endemic Medicinal Plant *Lavatera Cashmeriana* Camb.

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ABSTRACT

Medicinal plants are essential source of bioactive compounds. *Lavatera cashmeriana*, an endemic medicinal plant of Kashmir Himalaya, has an extensive record for its usage in traditional folk medicine. The plants with pharmacological importance harbour promising endophytic fungi with the ability to produce bioactive compounds identical to the one produced by the host plant. The plant was evaluated for fungal endophytes, which may yield promising bioactive metabolites with a wider range of applications. In this study, a total of 294 fungal endophytes associated with the healthy tissues of leaf, stem and root were isolated from *Lavatera cashmeriana*. Molecular identification based on ITS1-5.8S ribosomal gene-ITS2 DNA sequence analysis assigned them into 12 genera (16 species) belonging to 4 taxonomic orders of phylum Ascomycota. *Alternaria alternata*, *Pithomyces chartarum* and *Nigrospora oryzae* were found to be the predominant having overall colonization frequencies of 14.17%, 11.67% and 6.94% respectively, while as *Nectriaceae* sp. turned out to be least isolated species with an overall colonization frequency of 2.50%. The roots exhibited highest colonization frequency and species diversity was maximum in leaves.The study revealed that a higher diversity of endophytic fungi belonging to Ascomycota are associated with *L. cashmeriana*, and dominated by *Alternaria*. The endophytic fungi varied considerably among the plant tissues, with some fungal endophytes restricted to specific tissues only.

Keywords *Lavatera cashmeriana*, *Fungal-endophytes, Molecular Phylogeny, Colonization frequency, Diversity*.

INTRODUCTION

Kashmir Himalaya, an integral part of the Himalayan biodiversity hotspot, is known to harbour enormous diversity of endemic, rare and endangered medicinal plants (Dar and Khuroo, 2020; Hafeez *et al*., 2023). Medicinal plants have been reported to harbor distinct and rare micro-organisms that mimic the chemistry of their respective hosts and synthesize identical compounds or derivatives that are more bioactive than those produced by the host plants themselves (Strobel and Daisy, 2003; Suryanarayanan *et al*., 2009). Microorganisms such as endophytic fungi colonise in healthy tissues of plants asymptomatically and provide effective protection to the host against biotic and abiotic stresses (Kharwar *et al*., 2008). They are a vital source of naturally occurring bioactive compounds, playing tremendous role in microbial ecology. Endophytes are used for the discovery of new drugs and thus serve as a viable alternative in replacing plants and consequently conserving them (Sun and Guo, 2012; Teiten *et al*., 2013; Rajamanikyam *et al*., 2017). Only some of the medicinal plants of the Kashmir Himalayas have been little investigated both in terms of their endophytic fungal assemblages and the ability of endophytic fungi to produce bioactive metabolites of pharmaceutical importance (Nisa *et al*., 2018;

Jan *et al*., 2022; Wani *et al*., 2022). Therefore, further research on bioprospecting of endophytic fungi associated with medicinal plants is direly needed to discover novel endophytic microorganisms and their bioactive molecules from the region.

Lavatera cashmeriana Camb. (Kashmir Mallow or Wild Hollyhock) belonging to Malvaceae is an important endemic and endangered medicinal plant of Kashmir Himalaya (Mir *et al*., 2017; Wani *et al*., 2018; Majid *et al*., 2021; Akhter *et al*., 2025). The plant has been widely used both in the traditional and modern system of medicine (Wani et al., 2018). The plant extract is used locally due to its anti-inflammatory, analgesic and antibacterial activity (Rakashanda *et al*., 2013; Wani *et al*., 2018). The plant is used as a mild laxative in pregnant women, for treating throat problems (Aijaz *et al*., 2013), skin irritation (Mir, 2014), and common cold and mumps (Malik *et al*., 2011). Moreover, the seeds of the plant have been reported to act as protease inhibitors and have anti proliferative activity against human lung cancer cell lines (Rakhshanda *et al*., 2013). Despite its tremendous medicinal importance, the endophytic fungal communities of the plant have not been explored yet, and as such both the fungal endophytes and their chemical diversity in this medicinally important plant remains uncharacterized. It is in this backdrop, the present work was undertaken to study the diversity of endophytic fungi isolated from *Lavatera cashmeriana*, and the identification of isolates by molecular phylogeny using Internal Transcribed Spacer (ITS) as well as by colony morphology and microscopy.

MATERIAL AND METHODS

Plant material and study site

Fresh, healthy, and matured plants of *Lavatera cashmeriana* Camb. were collected from specific locations in Gulmarg (34˚02.670'N 74˚23.020'E, altitude of about 3200m) and Aharbal (33°38.281'N 74°46.653'E, altitude of about 2400m) regions of Jammu and Kashmir, India. The plant materials were transported to the laboratory in sterile polythene bags and stored at 4°C until processed. The collected plants were identified based on leaf, stem, and flower morphology by a taxonomist from the Centre for Biodiversity and Taxonomy, Department of Botany, University of Kashmir. The specimen was submitted to KASH herbarium under voucher specimen no.3727-KASH herbarium, Centre for Biodiversity and Taxonomy, Department of Botany, University of Kashmir, India.

Isolation and purification of culturable fungal endophytes

The surface treatment of samples and isolation of endophytic fungi was carried out as per the protocol of Petrini *et al*. (1992). The effectiveness of the surface sterilization process was checked according to the method described by Schulz *et al*. (1993). Plant samples were initially washed under running tap water followed by sterile distilled water (SDW) to remove soil particles and epiphytic microorganisms. Samples were immersed in 70% ethanol for 1-2 min and were surface sterilized with 4% sodium hypochlorite (NaOCl) for 2-3 min. Then the samples were washed with sterile distilled water 3-4 times at least for 3 mins to remove the traces of sodium hypochlorite. After this samples were rinsed again with 70% ethanol for 30 seconds. Samples were finally washed 4 times with sterile distilled water (SDW) in order to remove traces of ethanol and surface sterilants. The excess water was drained by placing the plant material on sterile filter paper. Surface drying was done under the sterile laminar airflow and the samples were cut into smaller segments of around 0.5 cm using a sterile scalpel. The surface sterilized samples of roots, leaves, and stems were incubated, at 28℃ for 1-3 weeks, on Potato Dextrose Agar (PDA) medium supplemented with 100 mg/ml streptomycin sulphate to prevent bacterial growth. The colonies were allowed to grow at the prescribed temperature, followed by sub culturing and pure culturing (Domsch *et al*., 2007). To check the effectiveness of surface sterilization, the surface sterilized material was directly implanted on PDA plates (Schulz *et al*., 1993). The pure cultures thus obtained were stored in a refrigerator at 4°C for future studies.

Colony morphology and microscopy of culturable endophytic fungi

The culturing of fungi was done on PDA (Merck; Cat No.: P2182) for 7 days at 28℃ and were observed daily for hyphal growth. Hyphal tip method was used for the transfer of colonies to fresh PDA plates for pure culture. Fungal colonies were transferred to the agar slant culture medium after sub-culturing and pure culturing for further use. Initially, colonies were identified based on colony morphology and colony color, fruiting structure, and sporulation (Crous *et al*., 2009). Macroscopic examination of the isolated fungal species was studied following the monographs for fungal identification (Domsch *et al*., 1980; Watanabe 2002). Slide culture technique was used for the preparation of microscopic slides, using lactophenol cotton blue staining (Kiffer and Morelet, 2000), and were then examined under a bright-field and phase contrast microscope. Several fungal characteristics were visualized and characterized under a compound microscope and a semiautomatic image analysis system on Olympus IX 71 inverted microscope (Olympus Optical, Tokyo, Japan), charge-coupled device (CCD) camera (Sony, Cambridge, UK), and the image analysis software installed was DP2-BSW ver. 2.1 for microscopic image processing. Light images of fungi were taken by objective lenses with 10X, 40X and 100X magnifications (Riddel, 1950; Harris, 1986).

Molecular identification of endophytic fungi

DNA extraction and Polymerase Chain Reaction (PCR) amplification

Genomic DNA extraction was done by using modified procedures from Allen *et al*. (2006). Endophytic fungi grown on PDA (pure culture) for 7 days were used for the extraction of genomic DNA using the standard manual and Genomic extraction kit (E5038; MERCK, Sigma Aldrich). Electrophoresis on 0.8% agarose gel stained with 0.5 µg/ml ethidium bromide (EtBr) was done to review the quantity and quality of DNA and visualized by UV trans-illumination. The DNA content and ratio was read using a NanoDrop reader (Thermo Fisher Scientific), which showed an A260/280 ratio of 1.8-2.0 and concentrations of 80 ± 5µg/ml. Polymerase chain reactions (PCR) using different primer regions were done. The primer pairs used for amplification are: ITS1 forward (5'TCCGTAGGTGAACCTGCGG3') and ITS4 reverse (5'TCCTCCGCTTATTGATATGC3') (White *et al*., 1990); NS1 forward (5'GTAGTCATATGCTTGTCTC3') and LR6 reverse (5'CGCCAGTTCTGCTTACC3') (Schoch *et al*., 2012). The polymerase chain reaction (PCR) conditions for the ITS1-5.8S-ITS2 gene segment were as follows: 95°C for 5 min, 33 cycles of 95°C for 1 min, 58°C for 1.5 min, and 72°C for 1 min, and a final elongation step of 72°C for 5 min. For 18S-ITS-28S, a final elongation step of 72°C for 10 min was performed to complete amplification. This was followed by 33 cycles of 95°C for 1 min, 58°C for 1.5 min, and 72°C for 1.5 min (Magray *et al*., 2021). The resulting PCR products were visualised in the 2% agarose gel to confirm the presence of a single amplified band and the bands were compared to the reference DNA ladder. Purification of PCR product was done by the ExoSAP-IT procedure from Thermo Fisher Scientific, India using the Big Dye Terminator sequencing kit (v3.1) and the purified product was sequenced by Barcode Sciences, Bangalore, India, and AgriGenome Labs in Kerala, India. The sequences were submitted to GenBank, National Centre for Biotechnology Information's (NCBI) for the allocation of accession numbers. The barcode gene markers were compared with reference gene sequences from the National Centre for Biotechnology Information's (NCBI) GenBank database using the basic local alignment search tool (BLASTn) (www.ncbi.nlm.nih.gov/ BLAST). The BLASTn tool was used to compare the acquired sequences with the sequences in GenBank in order to assess the taxanomic relatedness (Hafeez *et al*., 2023).

PHYLOGENETIC ANALYSIS

Sequences from the closely related organisms were downloaded to construct the phylogenetic trees using software MEGA X (Kumar *et al*., 2018). Sequences with the homology of 99 to 100% were obtained in the FASTA format from the NCBI database. The nucleotide sequences were aligned with the relevant or reference genus from GenBank using MAFFT 7.130 (Katoh and Standley, 2013). The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993) and boot strapping was carried out using 1000 replications. Initial tree(s) for the heuristic search with the highest log likelihood (-8328.24) were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 38 nucleotide sequences.

ECOLOGICAL METHODS FOR DIVERSITY ANALYSIS

The Shannon Weiner diversity index (H'), Pielou’s evenness index (J), Simpson’s diversity index (1-D), and inverse Simpson’s Index (1/D) were used to evaluate the diversity of endophytic fungi isolated from *Lavatera cashmeriana*. These diversity indices were used to understand fungal distribution and diversity in various plant parts.

STATISTICAL ANALYSIS OF THE ENDOPHYTIC FUNGI

All of the experiments were run in triplicates. In this study, the values are presented as mean ± SD. The diversity indices were calculated in R environment (R Core Team, 2024), using the *vegan package* v2.6-4 in R v.4.2.2 (Oksanen *et al*., 2022).

RESULTS

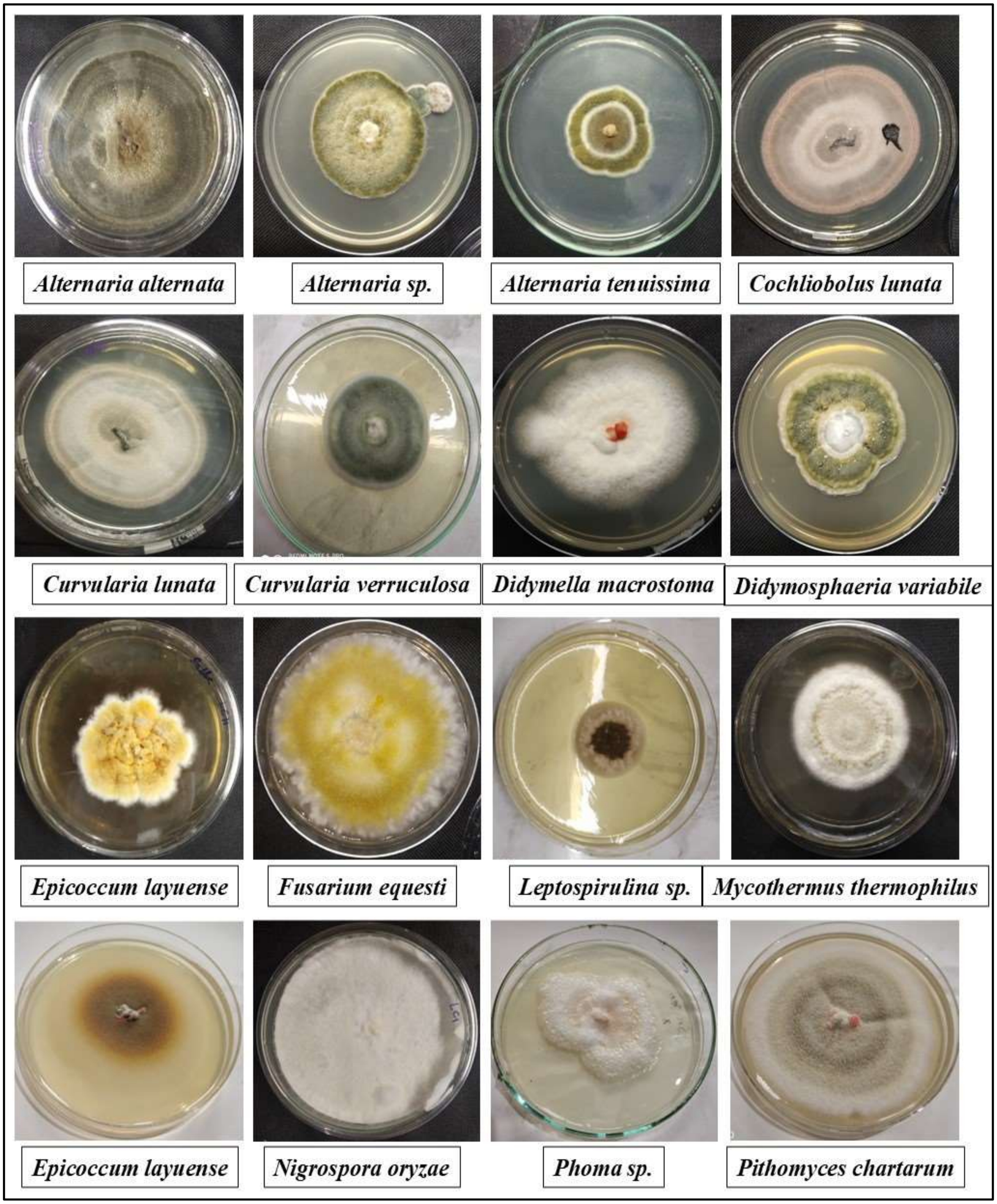
FUNGAL ENDOPHYTE DIVERSITY AND DISTRIBUTION

A total of 294 fungal isolates were obtained from *Lavatera cashmeriana*, of which 128 isolates were isolated from roots, 104 from leaves and 62 from stem. The number of endophytic fungi in roots were predominantly higher than those in stem, indicating that the fungal endophyte distribution varies considerably among the various plant tissues. Numerous isolates had the same morphological features. 55 isolates were identified on the basis of micro-morphology and then selected for molecular identification. The identified endophytic fungi belonged to 12 genera and 16 species of phylum Ascomycota Pl 1. *Alternaria alternata* was found to be the dominant species (51 isolates), followed by *Pithomyces chartarum* (42 isolates) and *Nigrospora oryzae* (25 isolates). Moreover, most of the fungal endophytes were isolated from all the plant tissues (i.e., root, stem and leaves) except for *Nectriaceae* sp., *Didymella macrostoma* and *Didymosphaeria variabile* collected from root and leaf tissues only, and *Phoma* sp. collected from leaf and stem tissues only. Dothideomycetes were dominant among the fungal endophytic population followed by Sordariomycetes. Pleosporales of Dothideomycetes were most common endophytic fungi in *Lavatera cashmeriana*. The relative frequencies of *Alternaria alternata* and *Pithomyces chartarum* in Pleosporales was 17.34% and 14.28% respectively (Table 1).

Diversity of endophytic fungi varied in different tissues of *Lavatera cashmeriana* (Table 1). The endophytic fungal richness was greater in the leaf (16 species) followed by root (15 species) and stem (13 species) tissues of the *Lavatera cashmeriana* (Fig. 1). The Shannon Weiner diversity index (H') and Simpson's diversity index (1-D) showed that the endophytic fungal diversity was higher in the roots (H' = 2.57 and 1-D = 0.91), followed by leaf (H' = 2.53 and 1-D = 0.90) and stem tissues (H' = 2.42 and 1-D = 0.89) (Fig. 2. A & B), thereby reflecting a relatively greater preference towards root colonization by fungal endophytes in *Lavatera cashmeriana*. Once again, the inverse Simpson's index (i.e., 1/D) was higher for root (1/D = 11.46) than leaf (1/D = 10.38) and stem (1/D = 9.56) tissues (Fig. 2C). Furthermore, the Pielou's evenness index (J) was higher for roots (J = 0.95) than stem (J = 0.94) and leaf (J = 0.91) tissues (Fig. 2D), thereby indicating relatively more even distribution of fungal endophytic isolates in roots than other tissues. Table 2 summarizes the diversity indices related to fungal endophytes obtained from *Lavatera cashmeriana,* indicating high species richness, diversity and evenness of different species. All endophytic fungi were isolated from three different tissues and thus could represent the entire fungal community.

Table 1: Diversity and phylogenetic affiliations of endophytic fungi isolated from roots, stems and leaves of Lavatera *cashmeriana*

Isolate ID	Phylum	Class	Order	Family	Genus	Closest Taxonomic Affinity (Accession no.)	Similarity %	GenBank accession no.	Number of isolates (relative frequency %)
LC1	Ascomycota	Sordariomycetes	Trichosphaeriales	Trichosphaeriales	*Nigrospora*	*Nigrospora oryzae* (AB220234)	98.32%	OK577960	25(8.5%)
A5			Hypocreales	Nectriaceae	*Fusarium*	*Fusarium equiseti* (KX463032)	100%	OP872649	22(7.48%)
A4					*Nectriaceae sp.*	*Nectriaceae sp.* (OP339687)	99%	OP872654	9(3.06%)
LC10			Sordarialres	Chaetomiaceae	*Mycothermus*	*Scytalidium thermophilum* (HQ221581)	99%	OK577964	11(3.74%)
LC8		Dothideomycetes	Pleosporales	Didymellaceae	*Phoma*	*Phoma sp.* (MT150595)	97.79%	OP862362	12(4.08%)
A2					*Didymella*	*Didymella macrostoma* (MT649577)	100%	OP862425	12(4.08%)
A3					*Leptosphaerulina*	*Leptosphaerulina sp.* (MW362328)	100%	OP872650	14(4.76%)
A9					*Epicoccum*	*Epicoccum layuense* (MT573479)	100%	OK577958	16(5.44%)
LC14				Pleosporaceae	*Curvularia*	*Curvularia verruculosa* (JN116709)	100%	OP895694	12(4.08%)
LC13						*Curvularia lunata* (MG661740)	98.38%	OK577962	17(5.78%)
LC11						*Cochliobolus lunata* (JN941608)	96.93%	OP862112	15(5.1%)
LC2					*Alternaria*	*Alternaria tenuissima* (ON237471)	100%	OP862110	12(4.08%)
LC12						*Alternaria sp.* (KJ489375)	97.87%	OK577963	13(4.42%)
A1						*Alternaria alternata*(MT644140)	100%	OK577957	51(17.34%)
A10						*Alternaria alternata* (OP886854)	100%	OK577959	
A6					*Pithomyces*	*Pithomyces chartarum* (MH859914)	100%	OK577961	42(14.28%)
LC6				Didymosphaeriaceae	*Didymosphaeria*	*Paraconiothyrium variabile* (KM096136)	96.80%	OP862361	11(3.74%)



Photoplate 1: Cultures of the endophytic fungal isolates obtained from *Lavatera cashmeriana*

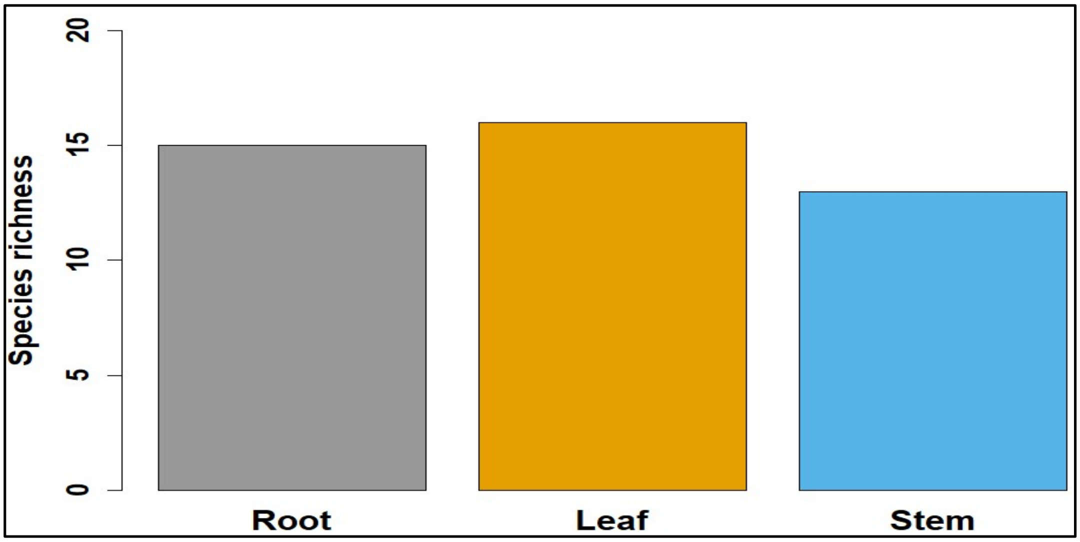


Fig. 1. Endophytic fungal richness among the tissues of *Lavatera cashmeriana*

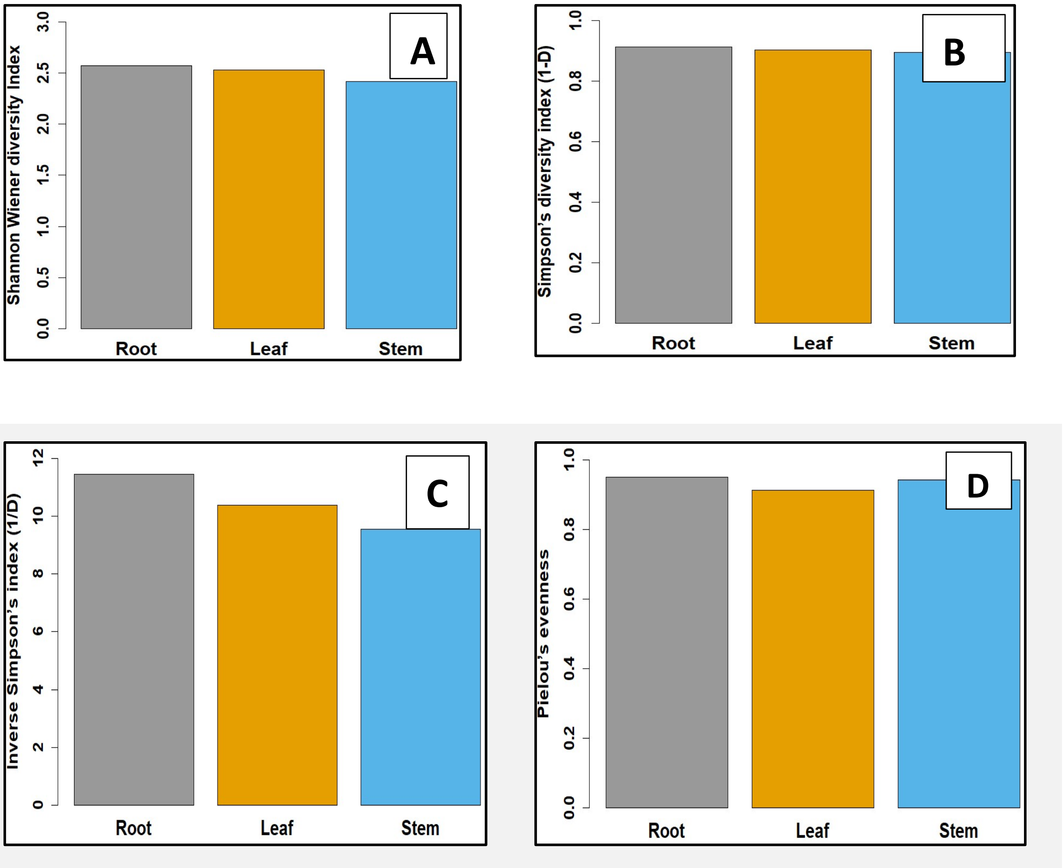


Fig. 2. The diversity indices for the endophytic fungi of *Lavatera cashmeriana* were represented as:

A) Shannon-wiener diversity index, B) Simpson's diversity Index, C) Inverse Simpson's index, D) Pielou's evenness Index.

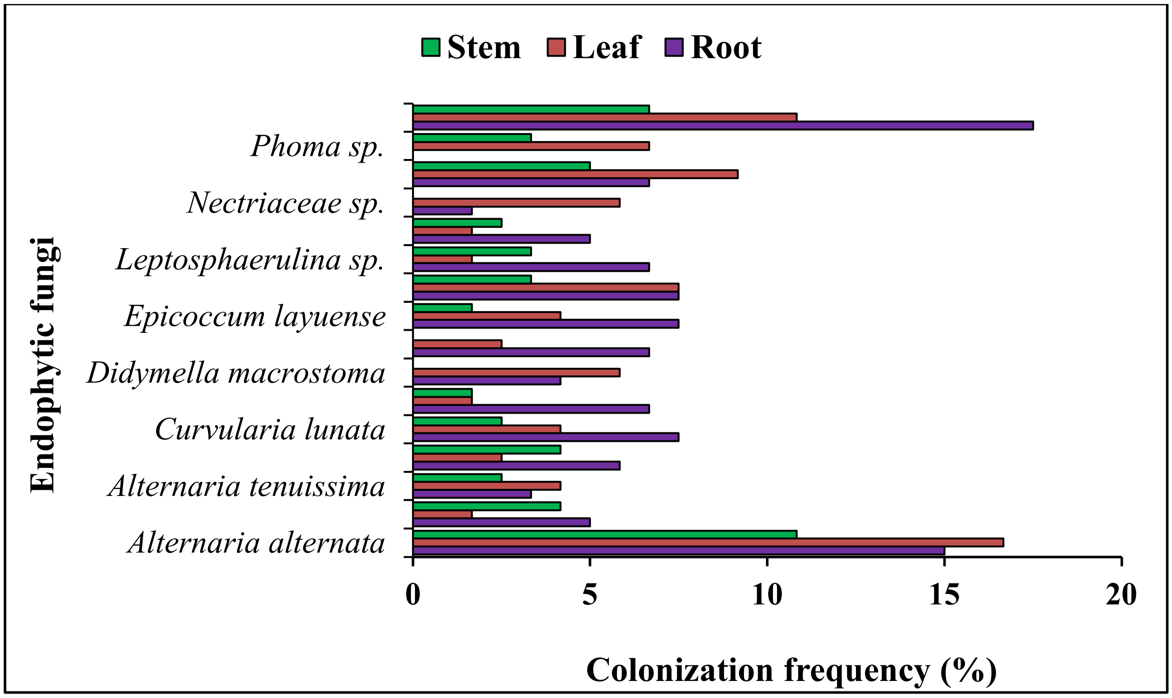


Fig. 3. Colonization frequency of fungal endophytes isolated from different tissues of *Lavatera cashmeriana*

Table 2. Diversity indices analyses of fungal endophytes

Diversity index	Different Tissues		
	Root	**Leaf**	**Stem**
Species richness	15	16	13
Shannon-Wienner Index	2.57	2.53	2.42
Simpson diversity Index	0.89	0.90	0.89
Inverse-Simpson Index	11.46	10.38	9.56
Pielou Index	0.95	0.94	0.91

Among the identified endophytic fungi, *Alternaria alternata*, *Pithomyces chartarum*, *Nigrospora oryzae* and *Fusariun equiseti* were found to be the predominant having overall colonization frequencies of 14.17%, 11.67%, 6.94% and 6.11% respectively, while as *Nectriaceae* sp. turned out to be least isolated species with an overall colonization frequency of 2.50% (Fig. 3). *Pithomyces chartarum* and *Alternaria alternata* were the predominant endophytic fungal species in the roots with a colonization frequency of 17.50% and 15% respectively (Fig. 3). Similarly, *Alternaria alternata*, *Pithomyces chartarum* and *Nigrospora oryzae* were predominant endophytic fungi in the leaves and stems with a colonization frequency of 16.67%, 10.83% and 9.17%, and 10.83%, 6.67% and 5% respectively (Fig. 3). This result in turn suggests the tissue specificity exhibited by the identified endophytic fungi. Some of the key characters related to colony formation, mycelium type, hyphae, and conidial structures for some of the representative fungal isolates are presented in Table 3.

Table 3. Macro- and micro-morphological observations and characteristics of fungal endophytes isolated from *Lavatera cashmeriana*

Isolate ID	Fungal Endophytes	Macro and Microscopic Characteristics
LC1	*Nigrospora oryzae*	Woolly greyish colony, conidiophores slightly branched conidia ellipsoidal, pale yellow.
A5	*Fusarium equiseti*	Colony multiplying, abundant aerial mycelium floccose. Initially, white colonies and reverse pale. Hyphae are smooth and branched. Conidia oval, hyaline, and septate.
A4	*Nectriaceae sp.*	Dense aerial mycelium with dark blond to buff towards the margin, zonation absent. Conidia septate.
LC10	*Mycothermus thermophilus*	Colonies off white. Hyphae colorless, septate; Conidia dark brown, smooth walled, globose to oval.
LC8	*Phoma sp.*	Colonies spread fast, greyish-brown, powdery, or suede-like; and abundantly aseptate small-sized, cylindrical, one-celled, and hyaline conidia.
A9	*Epicoccum layuense*	Colonies are multiplying fast, aerial floccose, bright yellow with yellow to pale brown reverse, having brown concentric rings near the center. Conidia are subglobose, dark brown, and hyphae-septate, branched.
LC14	*Curvularia verruculosa*	Flattened grayish-color colony and black pigmentation on the back side of the plate. Hyphae smooth and branched, thick-walled. Conidia verrucose and mostly curved.
LC13	*Curvularia lunata*	Colonies are fast-growing, black to downy, brown to blackish brown with a black reverse. Conidia smooth-walled, olivaceous brown.
LC11	*Cochliobolus lunata*	Brown to black colour, hairy, velvety or woolly texture, and loosely arranged and rapidly growing colonies. Conidia smooth, 3- septa and 4 cells.
LC6	*Didymosphaeria variabile*	Most of the colony surface covered by dense woolly-floccose, first whitish or pale primrose, then olivaceous aerial mycelium; hyphae hyaline to pale brown, septate, smooth; Conidia dark to light brown.
LC12	*Alternaria tenuissima*	The fast growing colonies with olive dark ash colored, gray aerial hyphae, and wolly texture. Conidia are long solitary or in short unbranched chains (three to five conidia), golden brown to brown, and smooth-walled with septa.
LC2	*Alternaria sp.*	Colonies are fast-growing, black to olivaceous-black or greyish, and are suede-like to floccose. Microscopically, branched acropetal chains of multicellular conidia. Conidia are obclavate, pale brown, smooth-walled, or verrucose.
A1, A10	*Alternaria alternata*	Colonies are fast growing, black to olivaceous black or greyish with a very thin white margin and cottony texture. Branched acropetal chains of multicellular conidia, obclavate, sometimes ovoid or ellipsoidal, pale brown, smooth walled.
A6, A11	*Pithomyces chartarum*	Fast-growing colonies, suede-like to downy and black. Conidia are medium to dark brown; septate.
A2	*Didymella macrostoma*	Colonies are smooth, velvety-fluffy with sparse, colorless mycelium. Conidia are irregular, ellipsoidal, smooth, and hyaline. Conidia is usually aseptate but sometimes single septate.
A3	*Leptosphaerulina sp.*	Colonies brown to dark brown, white to pale yellow at the edge, sometimes dark grey, covered by white fluffy hyphae, flattened; Conidia initially hyaline mostly septate.

Woolly greyish colonies with slightly branched conidiophores bearing pale yellow and ellipsoidal conidia were formed by isolate *Nigrospora oryzae* LC1. The multiple colonies having abundant floccose aerial mycelium were seen for isolate *Fusarium equiseti* A5. The colonies were white initially and turn peach orange at agar base to buff-brown; reverse pale. Micro-morphologically the hyphae were smooth, branched, cylindrical, septate, and hyaline with oval, hyaline, and septate conidia. For the isolate *Nectriaceae sp.* A4, the colonies had dense aerial mycelium with dark blond to buff towards the margin, zonation is absent and conidia were septate and ellipsoid to sub-cylindrical in shape. The isolate of *Mycothermus thermophilus* LC10 formed colonies appearing whitish at first and then turned black gradually. Micro-morphologically, the hyphae appeared colourless and septate. The conidia were intercalary, dark brown, smooth walled, globose to oval. The *Phoma sp.* LC8 isolate formed colonies that spread fast, were greyish-brown, powdery, or suede-like; and produce abundantly aseptate small-sized, globose to cylindrical, one-celled, and hyaline conidia. The isolate *Epicoccum layuense* A9, also produced fast multiplying colonies that were aerial floccose, bright yellow with yellow to pale brown reverse, having brown concentric rings near the center. Conidia were sub-globular, verrucose, smaller epi-coccoid, dark brown, and hyphae are septate and branched. The isolates of *Curvularia verruculosa* LC14, *Curvularia lunata* LC13, and *Cochliobolus lunata* LC6 appeared flattened grayish-color colony with black pigmentation on the back side but the anomalies in these isolates were seen at conidiophores. For LC14, the conidia were verrucose, mostly curved with three septa, light to dark brown, multicellular with the middle third cell larger and darker than the other three remaining cells. For LC13, the conidia were smooth-walled, olivaceous brown with end cells somewhat paler; and having obovoidal to broadly clavate conidia, curved at the subterminal cell, septate, with the subterminal cell swollen and distinctly larger than the remaining cells. For LC6 the conidia were hyaline initially, but mature conidia appeared dark to light brown, small cylindrical to ellipsoidal with both ends obtuse, single celled, smooth walled to fine verruculose and thin-walled. The isolates of *Alternaria* sp. were fast-growing colonies that were black to olivaceous-black or greyish, and are suede-like to floccose or with grey aerial hyphae, and woolly texture. The conidia were long solitary or in short unbranched chains (three to five conidia), golden brown to brown, obclavate or elliptical tapering into a slightly swollen beak, and smooth-walled and septate. The *Pithomyces chartarum* A6 and A11 isolates also formed fast-growing colonies that are suede-like to downy and black. The conidiophores are pale olive, smooth, or verrucose bearing medium to dark, septate brown, with one or both median cells divided by longitudinal septa. The *Didymella macrostoma* A2 isolate form smooth colonies appearing velvety-fluffy with sparse, colorless mycelium passing into the conidiophore. The conidia are irregular, ellipsoidal, smooth, and hyaline and usually aseptate but sometimes single septate. Finally, the *Leptosphaerulina sp.* A3 isolate forms brown to dark brown colonies, appearing white to pale yellow at the edge, but sometimes dark grey, covered by white fluffy flattened hyphae. The conidia are initially hyaline becoming brown to dark brown when mature, having oblong to cylindrical or ellipsoidal shape and are mostly septate.

Molecular identification and phylogenetic relationship of fungal endophytes

Based on the PCR amplification, we obtained various amplified products having specific lengths including ITS1-5.8SrRNA-ITS2 (500bp approx.), LSU (1000bp approx.), SSU (1000bp approx.), and SSU-ITS1-LSU (1500 approx.). Using the molecular sequence data, we adopted the phylogenetic analytical methods to overcome the limitations inherent in macro- and micro- morphological characterization for fungal species identification. Using sequences from the GenBank, NCBI, we found that the Internal Transcribed Spacers (ITS) and ribosomal RNA genes showed 99–100% match with the related fungal species. The detailed records of the accession codes from the NCBI for different isolates, gene and species name are presented in Table 1. The phylogenetic analysis confirmed the results of the morph-taxonomic features, thus revealing a total of 16 endophytic fungal species belonging to phylum Ascomycota.

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model from our LSU database. The tree with the highest log likelihood (-8328.24) is shown in Fig. 4. This analysis involved 38 nucleotide sequences. *Alternaria alternata* A10 (OK577959) and A1 (OK577957); were isolated and aligned alongside additional *A. alternata* species from GenBank accession numbers OP886854 and MT994749 in the Maximum Livelihood tree of LSU sequences. The *Curvularia verruculosa* OP895694 from *Lavatera cashmeriana* showed a similarity to the *C. verruculosa* JN116709 strain and *C. americana* MH037565 strain. The third clade in Fig. 4 shows that the *Leptosphaerulina* sp. OP872650 sequence from our LSU database had the highest similarity (100%) to other sequences OP810862 and MW362328 obtained from the GenBank database (LSU tree).

Similarly, *Nectriaceae* sp. OP872654 branched off as member of fourth clade displayed 100% similarity to OP339687 *Nectriaceae* sp. and *Fusarium equiseti* OP872649 showed high similarity to *F. equiseti* (KX463032) and *F. equiseti* (MW945400). The sequences of *Alternaria tenuissim*a LC2, OP862110 separated into a clade of other *Alternaria alternata* B8 isolate and *Alternaria tenuissima* NFUA02 isolate. *Pithomyces chartarum* A6, OK577961 exhibited highest similarity with *Pseudopithomyces chartarum* MW361304 and *P. chartarum* MH859914. At the end of the tree the two isolates of *Curvularia* sp. LC10R and LC13R, branched off into a separate clade along with *Mycothermus thermophilus* and exhibited a high similarity with *Curvularia* sp. BCC52426. Although some of the sequences showed host specificity at the level of genera, however the GenBank sequences showing analogy with the current sequences were host independent and without any source specificity. Evolutionary history was inferred from the SSU tree with the highest log likelihood (-4113.88) shown in Fig. 5. This analysis involved 20 nucleotide sequences from five major clades. The sequence of *Didymosphaeria variabile* OP862361 split in a branch and showed high similarity with

KM096136 *Paraconiothyrium variabile* strain. *Nigrospora oryzae* LC1 exhibited similarity with MZ798388 *Nigrospora oryzae* from the SSU tree. The SSU sequences of *Phoma* OP862362 and *Didymella macrostoma* OP862425 separated along *Phoma* sp. AB252869 having smaller nucleotide sequences. The sequences of *Alternaria* sp. OK577963 separated in a clade of other *Alternaria alternate* isolate AB2. *Epicoccum layuense* A9 displayed 100% similarity with other *Epicoccum* sp.

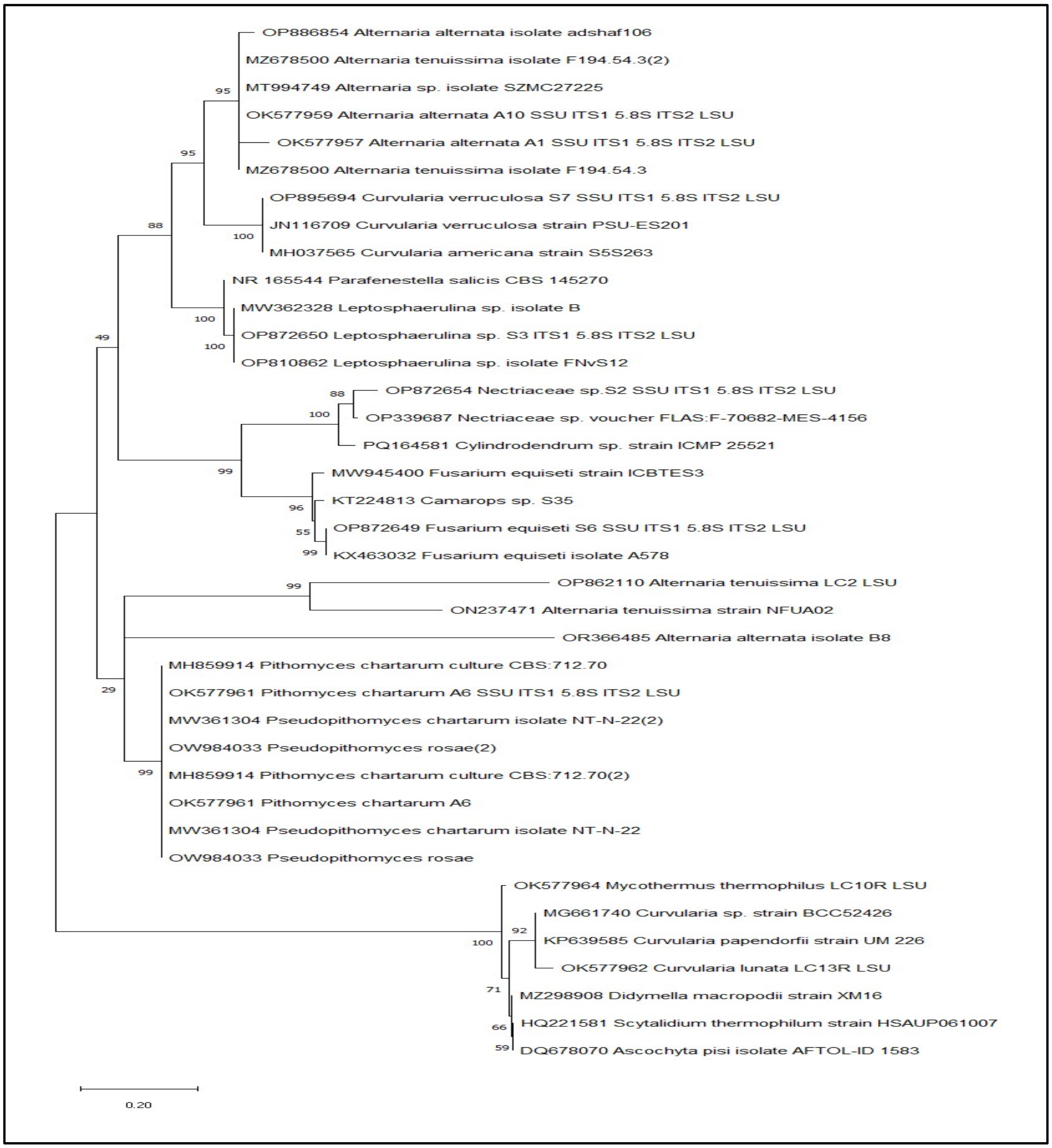


Fig. 4. Phylogenetic tree of the LSU sequences from the isolates of endophytic fungi derived from *Lavatera cashmeriana* based on 1000 boot-strap replications post to alignment by Muscle alignment option. The tree was constructed using the Maximum Likelihood method and following the Tamura-Nei model (Tamura and Nei, 1993).

Shown is the tree with the highest log likelihood (-8328.24). The percentage of trees having the associated taxa clustered together are presented next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in terms of the number of substitutions per site. This analysis involved 38 nucleotide sequences. A total of 1773 positions remained in the final dataset. The phylogenetic analyses were performed in MEGA X (Kumar *et al.*, 2018).

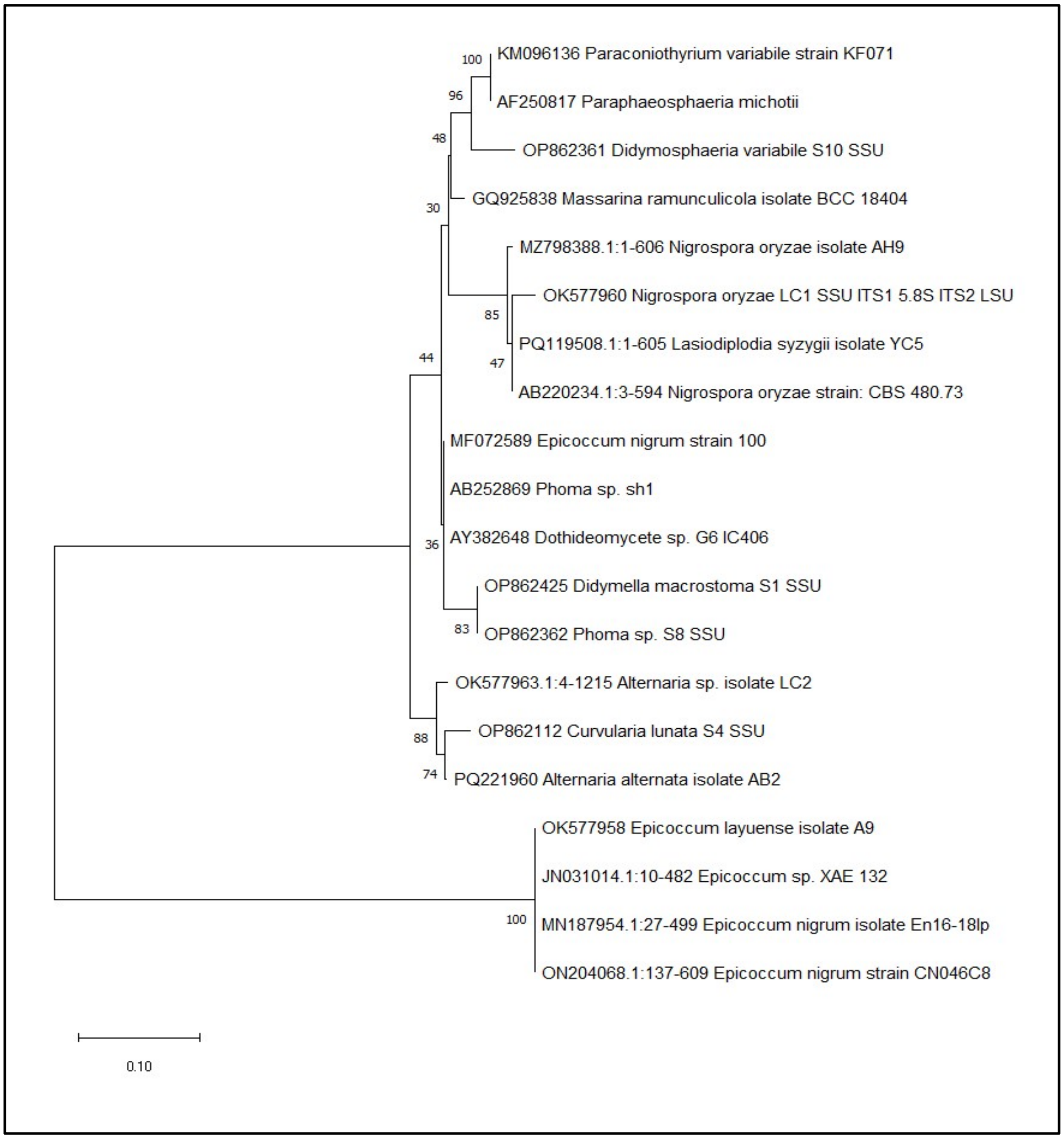


Fig 5. Phylogenetic tree of the SSU sequences from the isolates of endophytic fungi derived from *Lavatera cashmeriana* based on 1000 boot-strap replications post to alignment by Muscle alignment option. The tree was constructed using the Maximum Likelihood method and following the Tamura-Nei model (Tamura and Nei, 1993)

The percentage of trees having the associated taxa clustered together are presented next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in terms of the number of substitutions per site. This analysis involved 20 nucleotide sequences. The codon positions included were 1st+2nd+3rd+Noncoding. A total of 1423 positions remained in the final dataset. The phylogenetic analyses were performed in MEGA X (Kumar *et al*., 2018).

DISCUSSION

Over the last two decades, a plethora of research studies have been conducted to isolate and characterize fungal endophytes from medicinal plants and to explore their bioactive potential (Schulz *et al*., 2002; Strobel and Daisy, 2003; Kumar and Hyde, 2004; Yimgang *et al*., 2022). However, majority of such scientific studies have been carried out in developed countries especially from the Global North (Padhi *et al*., 2013). The scientific studies from developing countries including India are scarce (Bhardwaj and Agrawal, 2014; Desale, 2016; Devi *et al*., 2022). The endophytic fungi represent a diverse group of fungal diversity (Hafeez 2023), occurring in almost all plant tissues (Zheng *et al*., 2017). Owing to their symbiotic relationship with plants, the endophytic fungi help plants to cope up harsh environments and acts as potential bio-control agents against various plant pathogens (Zheng *et al*., 2017; Shen *et al*., 2019; Hafeez *et al*., 2023). The endemic plants with a long standing ethno-medicinal history provide a unique natural resource for discovering novel endophytic fungi, as the medicinal properties associated with such plants could be likely because of the inhabiting endophytic fungi (Strobel and Daisy, 2003; Kusari *et al*., 2013). However, such endophytic fungi are still understudied and poorly represented (Tejesvi *et al*., 2011). Thus, additional studies are direly needed to explore medicinal flora of with reference to endophytic fungi to minimize the over harvesting of such flora and protect their declining populations (Deka and Jha 2020; Du *et al*., 2020; Hafeez, 2023).

Lavatera cashmeriana Camb. being one of the important endemic and endangered medicinal plant of Kashmir Himalaya was selected for endophyte isolatin based on its endemic status and medicinal potential. Despite its tremendous medicinal importance, the endophytic fungi associated with the plant and their bioactivity are yet to be characterized. To our best knowledge, this work for the first time reported the diversity and bioactivity of endophytic fungi associated with *Lavatera cashmeriana*.

Our study found a higher diversity of endophytic fungi associated with *Lavatera cashmeriana* which align with several published studies that documented the exploration of diverse endophytic fungi specifically from medicinal flora (Du *et al*., 2020; Hafeez, 2023). In this study, all the endophytic fungi reported from *Lavatera cashmeriana* belonged to Ascomycota. This finding is in accordance with several studies (Chowdhary and Kaushik, 2015) *Ocimum sanctum*; (Raja *et al*., 2015) *Silybum* *marianum*; (Zhou *et al*., 2018) *Rhizophora stylosa* and *R. mucronata*; (Rai *et al*., 2022a) *Oroxylum indicum*, wherein the identified endophytic fungi were from Ascomycota only. The possible explanation for this is that Ascomycota represents the largest, most common, and widely distributed phyla of endophytic fungi covering nearly 8% of the total earth's land surface and thus can be easily identified following universally accepted standard isolation protocols (Schoch *et al*., 2009; Mishra *et al*., 2016; Pecoraro *et al*., 2018). Most of the isolated endophytic fungi were from the order Pleosporales, which is in line with several reports documented in literature (Wang *et al*., 2015) *Oryza rufipogon* ; *Securinega suffruticosa* (Du *et al*., 2020). *Alternaria* was found to be the dominant genera with three species. *Alternaria* has been documented as the predominant genera among endophytic fungi isolated from different medicinal plants (Sun *et al*., 2008; Huang *et al*., 2008; Gallo *et al*., 2009; Qadri *et al*., 2013; Hamzah *et al*., 2018). The most frequently isolated endophytic fungi in this study were *Alternaria alternata*, *Pithomyces chartarum* and *Nigrospora oryzae*. These endophytic fungal species have been documented to be frequently isolated from several other plants as well. Elghaffar *et al*. (2022) isolated *A. alternata* from the leaves of *Ziziphus spina-christi*, Chandra *et al*. (2021) obtained it from the *Picrorhiza kurroa*, Lee *et al*. (2019) isolated it from the *Vitex rotundifolia*, and Khiralla *et al*. (2016) isolated the same from Sudanese medicinal plants. Similarly, *Pithomyces chartarum* was isolated from *Lupinus luteus* by Garcia-Latorre *et al*. (2024), *Styrax sumatrana* by Elfiati *et al*. (2021), *Cissus quadrangularis* by Suradkar and Hande (2017), and *Butea monosperma* by Tuppad and Shishupala (2014). Likewise, *Nigrospora oryzae* was reported by Suradkar and Hande (2017) from *Cissus quadrangularis*, Ebada *et al*. (2016) from the *Loranthus micranthus*, and Hafeez *et al*. (2023) from the *Aconitum heterophyllum*. Other endophytic fungi isolated from *L. cashmeriana* in this study have also been isolated from several other plants. For example, *Fusariun equiseti* has been isolated from *Solanum tuberosum* (Mamaghani *et al*., 2024), *Alternaria tenuissima* from *Rhizophora stylosa* (Sun *et al*., 2013) and *Psidium guajava* (Chatterjee *et al*., 2022), *Curvularia lunata* from *Axonopus compressus* **(**Azuddin *et al*., 2023) and *Dioscorea bulbifera* (Sharma *et al*., 2023), *Didymella macrostoma* from *Fraxinus excelsior* (Barta *et al*., 2022) and *Brassica napu* (Cheng *et al*., 2024), *Curvularia verruculosa* from *Catharanthus roseus* (Parthasarathy *et al*., 2020), and *Didymosphaeria variabile* from *Cornus florida* (Maheshwari and Mmbaga, 2024).

The present study reveals that the endophytic fungal species varies considerably among the plant tissues, with some fungal endophytes restricted to roots only. The tissue specific exhibition by endophytic fungi has been widely reported in literature *Hyoscyamus muticus* (Abdel-Motaal *et al*., 2010); *Pteroceltis tatarinowii* (Chai *et al*., 2016); *Zanthoxylum bungeanum* (Li *et al*., 2016); *Securinega suffruticosa* (Du *et al*. 2020); *Aconitum heterophyllum* (Hafeez *et al*., 2023). The possible explanation for this tissue specificity could be the inherent differences in host plant tissues with respect to micro-environmental conditions, nutrient availability, anatomy and age (Maheshwari and Rajagopal, 2013; Zhou *et al*., 2018; Du *et al*., 2020). The endophytic fungal richness was greater in the leaf than other plant tissues. Similar results of leaves harbouring higher endophytic fungi were reported in several scientific studies (Barnett and Hunter, 1998; Suryavamshi and Shivanna, 2020; Jagadish and Chowdappa, 2021). Furthermore, the higher number of endophytic fungal were isolated from roots than leaves and stems. Such a trend of isolating maximum fungal endophytes from root than other plant tissues has been reported in several published studies (Fisher *et al*., 1995; Desale, 2016). The observed differences in the isolation of endophytic fungi from various plant tissues can be likely due to specific substrate utilization patterns developed by these tissues (Desale, 2016). Our study revealed that the diversity of endophytic fungi showed some amount of heterogeneity in different plant tissues, with the highest diversity observed in roots followed by leaves and stems. Endophytic fungi are found to be present in almost every tissue of plant. Highest root colonisation may be because of their symbiotic mycorhizal association. Consistent with our finding, Nisa *et al*. (2015) showed that the maximum endophytic fungal diversity occurs in the roots of *Cymbidium aloifolium*. The possible reason for the highest degree of isolation and diversity of endophytic fungi endophytic fungi in roots than other plant tissues can be exposure of roots to the rhizosphere mycoflora thus facilitating easier root colonization (Vasanthakumari and Shivanna 2009; Sarma *et al*., 2018). Furthermore, the greater endophytic fungal diversity in the leaves than stems as observed in the present study is supported by many previously published studies (Fisher *et al*., 1995; Kharwar *et al*., 2011). The possible explanation for this finding that the relatively wider leaf surface area exposed to environment favours the adherence and deposition of spores that are more likely to form endophytes with passage of time and arrival of favourable conditions (Fisher *et al*., 1995; Kharwar *et al*., 2010).

CONCLUSION

Considering the huge diversity of fungal endophytes and their biological activity, it becomes imperative to explore these endophytes both for the discovery of novel bioactive compounds and preserving the biodiversity and ethanopharmacological importance of medicinal plants. The results reveal that *Lavatera cashmeriana* has a diverse community of fungal endophytes associated with them, which may serve as a valuable source of bioactive compounds for future medical applications.

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