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Genetic Diversity of Fungal Endophytes

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Abstract: ***Piper nigrum is an important plant used in traditional medicine because of its metabolite richness and medicinal property. So the endophytic fungi associated with this plant can also expect to have promising bioactive metabolites. In this study, eighteen endophytic fungi were isolated from Piper nigrum and based on molecular and phylogenetic analysis, the isolates belong to the seven genera were identified. These include,* Colletotrichum *sp., Diaporthe sp., Phomopsis sp., Mycosporella sp., Fusarium sp., Pleosporales sp. and Pseudocercospora sp. The results of the study showed diversity of endophytic fungi associated with Piper nigrum.***

Keywords: *Piper nigrum*, endophytes, fungi, plant tissue, diversity, secondary metabolites

1. Introduction

Natural biodiversity rich of India can be considered as a 'gold mine' of diverse endophytic fungi with remarkable bioactive potential. Undoubtedly, extensive amount of studies have been conducted in India related to the field of endophytic fungi since 1998 (Hoque et al., 2023). In accordance with the available literature, more than 100 indigenous Indian medicinal plants have already been collected and their endophytic fungi identified. Hence more reasonably streamlined efforts are required in the field of endophytic fungal research for the identification of more bioactive compounds (Chithra et al., 2014, 2018).

Endophytic fungi is present inside the plant tissue without causing any symptoms of disease to the host plant (Toppo et al., 2024). The colonization of endophytes involves four major steps like host acceptance by the fungus, spore germination, penetration of epidermis and tissue colonization, the colonies in perennial plant parts may have a very long life (Stelmasiewicz et al., 2023).

The distribution and diversity of endophytic fungi is considerable to be depend on the taxonomy, genetic background, age, and tissues of the host plants, in addition to the type of environments (Iyabo et al., 2023). Endophytic fungi have been reported to support and their host plants through enhancement of the growth through increase in the resistance to biotic/abiotic stresses, and also accumulation of secondary metabolites produced originally by the host plant (Fazeli *et al*., 2024).

Many studies have reported endophytic fungi from traditional medicinal plants as source of promising bioactive compound with diverse pharmaceutical applications (Anand et al., 2023). Even though *Piper nigrum* is well known for its bioactive metabolites, the significance of endophytic fungi associated from the plant has not been reported earlier. Hence, the present study was carried out to identify the endophytic fungal community present in *P. nigrum*.

2. Materials and Methods

2.1 Selection of plant species

Healthy and mature stem pieces of various cultivars (Karimunda, Narayakodi, Kottanadu) of *Piper nigrum* collected from local farms were used for the investigation of endophytic fungal community.

2.2 Isolation of endophytic fungi

Surface sterilization procedure for the isolation of endophytic fungi was carried out as described by Chithra *et al., (*2018) with minor modifications. Plant samples were washed under running tap water for 10 min followed by immersion in 70% EtOH for 1 min and in NaOCl (2.5% available chlorine) for 10 min. This was then drained and immersed in 70% ethanol again for 30 sec. Finally, the samples were rinsed with sterile distilled water several times and the final wash was plated on to media as control. Plant samples were then cut aseptically into 1 cm long segments. The cut surface of the segments were placed on petri - dishes containing various media (**Table.1**) like Casamino acids Yeast extract Dextrose Agar (CYD), Arginine Glycerol Agar (AGA), Low Nutrient Mineral Salts Agar (LNMS), Potato Dextrose Agar (PDA) and Mineral Salt Starch Casein Agar (MSSC) amended with 50µg/mL nalidixic acid. The control and inoculated plates were incubated at 28°C for 5 days and observed for the fungal growth. The fungal isolates obtained were further purified on Potato Dextrose Agar medium. The isolates were initially subjected to staining and microscopic observation and were further identified by molecular methods.

Table 1: Media used for the isolation of endophytic fungi

Low Nutrient Mineral Salts Agar (LNMS) Modified from Reddi and Rao, (1971)	NaCl - 0.2 g $MgSO_4.7H_2O$ - 0.05g $CaCO_3$ - 0.02g $FeSO_4.7H_2O$ - 0.01g Starch - 0.1 g Yeast extract - 0.1g K_2HPO_4 - 2 g Agar - 18g Distilled water - 1000 mL

Arginine Glycerol Agar medium (AGA) Modified from EI - Nakeeb and Lechevalier, *(*1963)	L - Arginine - 1 g Glycerol - 12.5g NaCl - 1g $MgSO_4$. H_2O - 0.5g K_2HPO_4 - 1g Fe $(SO_4)_3.6H_2O$ - 0.01g $CuSO_4.5\ H_2O$ - 0.001g $ZnSO_4.7\ H_2O$ - 0.001g Agar - 18g Distilled Water - 1000 mL
Casamino acids/Yeast extract/Dextrose Agar (CYD) Modified from Jackson and Ball, (1994)	Cas Aminoacid - 0.5g Yeast extract - 0.8g D - glucose - 0.4g K_2HPO_4 - 2g Agar - 18g Distilled water - 1000 mL
Mineral Salt Starch Caesin Agar (MSSC) Modified from Reddi and Rao, (1971)	NaCl - 2g $MgSO_4.7H_2O$ - 0.05g $CaCO_3$ - 0.02g $FeSO_4.8\ H_2O$ - 0.01g KNO_3 - 2g Starch - 10g Casein - 0.3g K_2HPO_4 - 2g Agar - 18g Distilled water - 1000 mL
Potato Dextrose Agar (PDA) Modified from BAM media Composition	Potato - 200g Dextrose - 20g Agar - 20g Distilled water - 1000 mL

2.3 Morphological identification of endophytic fungi by Lactophenol cotton blue stain

For studying the morphological characters, the slide culture of the fungus was prepared and stained using lacto phenol cotton blue. For this, the potato dextrose agar blocks (7 × 7 mm) were aseptically transferred on a pre - sterilized glass slide placed inside a sterile petri dish. The block was inoculated with the fungal isolate. A cover slip was placed on the top of the agar block and was incubated for 5days at 30 ± 2◦C. After incubation the cover slip was retrieved and placed on a drop of lacto phenol cotton blue stain (Madavasamy and Panneer selvam, 2012). This was then observed under bright field microscope (Olympus, Model BX43) with 100× oil immersion objective and the images are processed with Q imaging software. Spore morphology and vegetative structures were compared with a fungal identification manual (de Hoog et al., 2000).

2.4 Molecular Identification of endophytic fungi

The genomic DNA from the endophytic fungi was isolated using Chromous Biotech Fungal gDNA Mini spin kit (Category number RKT41) according to the manufactures recommendation. The total genomic DNA was extracted from the mycelia of all the 18 isolates. For this, the fungi was inoculated into 50 mL Potato Dextrose Broth and incubated at room temperature for 5 days in an orbital shaker. After incubation, the pellet was collected from the fungal culture. Then 500 mg of fungal mycelia was added into the pestle containing 500 µL of 1X suspension buffer and crushed with mortar and pestle. The crushed fungal mycelia was transferred into 2 mL vial and mixed thoroughly. The mixture was then placed at 65ºC for 10 min and added 1 mL of lysis buffer. Then it was kept in water bath at 65ºC for 15 min and the mixture was centrifuged at 10000 rpm for 1min at room temperature. The content in the collection tube was discarded and the spin column was placed again in the collection tube. Then 500 µL of 1X wash buffer was added into the column and centrifuged at 10000 rpm for 1 min, discarded the contents and the empty column with collection tube was centrifuged at 10000 rpm for 3 min. The spin column was placed in the fresh vial and added 25 µL of warm elution buffer into it. Then it was kept at 65ºC for 1 min and centrifuged at 10000 rpm for 1 min at room temperature. Eluted DNA was collected in the same vial.

ITS specific primers were used for the PCR. The primers used were ITS1 (5′ - TCC gTA ggT gAA CCT gCg g - 3′) and ITS4 (5′ - TCC TCC gCT TAT TgA TAT gC - 3′) and were designed based on previous reports of White *et al*., (1990). PCR was performed at a final volume of 50 µL reaction containing 50 ng of genomic DNA, 20 pmoles of each primer, and 1.25 units of Taq DNA polymerase (Bangalore Genei), 200 µM of each dNTPs and 1 X PCR buffer. The PCR was performed with an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55.5°C for 2 min and extension at 72°C for 2 min with a final extension at 72°C for 10 min. The amplified product was analyzed using 1.2% (w/v) agarose gel, purified and was further used for DNA sequencing. The product was further purified for its use as the template for sequencing PCR using Big Dye Terminator Sequence Reaction Ready Mix (Applied Biosystem). The sequence thus obtained was further subjected to Basic Local Alignment Search Tool (BLAST) analysis. For the Phylogenetic analysis, related sequences were retrieved from NCBI and aligned with ClustalW. The aligned data was used for further phylogenetic analysis with neighbour - joining method using MEGA 5 with 1, 000 boot strap replicates

3. Results

After several rounds standardization of surface sterilization procedure, the isolation has resulted in the purification of 18 endophytic fungi (PF 1 - 18) from various cultivars of *Piper nigrum (***Table 2** and **Fig.1**). The absence of growth in the control plate ensured the proper surface sterilization of the used plant tissue and confirmed the isolated fungi as endophyte. The isolates were initially distinguished by the difference in colony characters and further by morphological features using staining techniques (**Fig.2**). The isolates with distinct characters were selected, purified and sub - cultured for maintenance as pure culture on PDA slants for further studies.

Table 2: Summary of the endophytic fungi isolated from different cultivars of *P. nigrum*

No	Name of cultivars	Fungi collected
1.	Karimunda (Idukki)	PF1 to PF9
2.	Narayakodi (Kottayam)	PF10 to PF14
3.	Kottanadan (Trivandrum)	PF15 to PF18

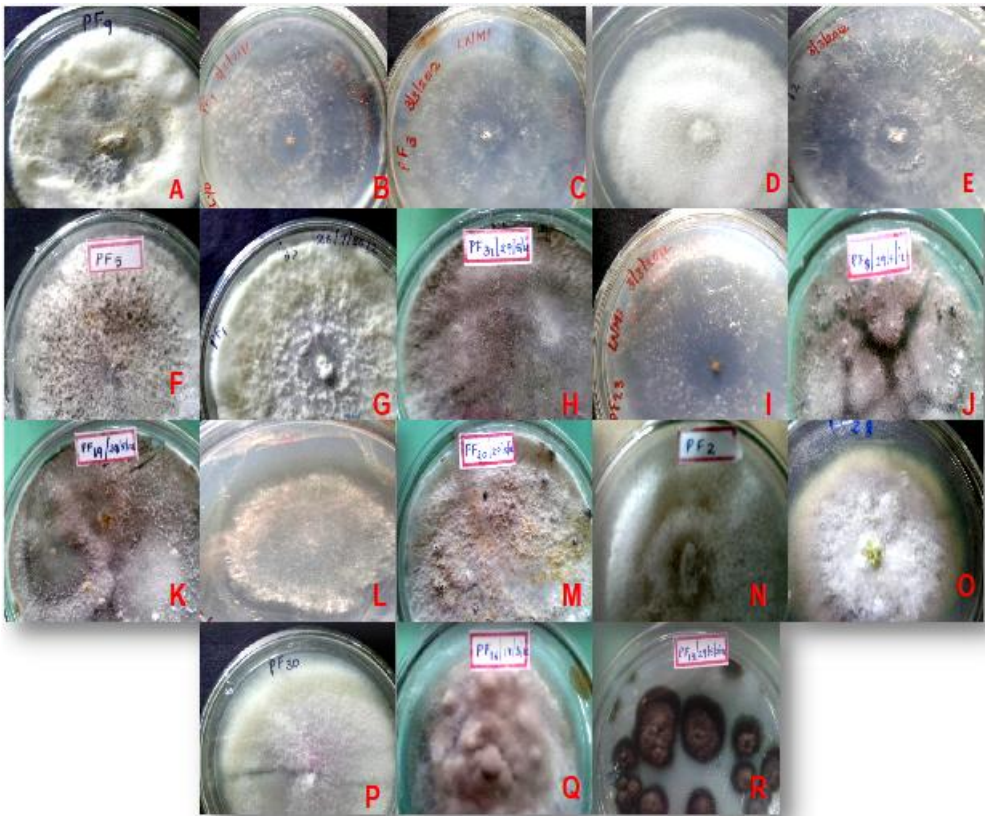

Figure 1: Endophytic fungi isolated from different cultivars of *Piper nigrum*. A – I – PF1 – PF9 (Karimunda); J – M – PF10 - PF14 (Narayakodi); N – R – PF15 - PF18 (Kottanadan)

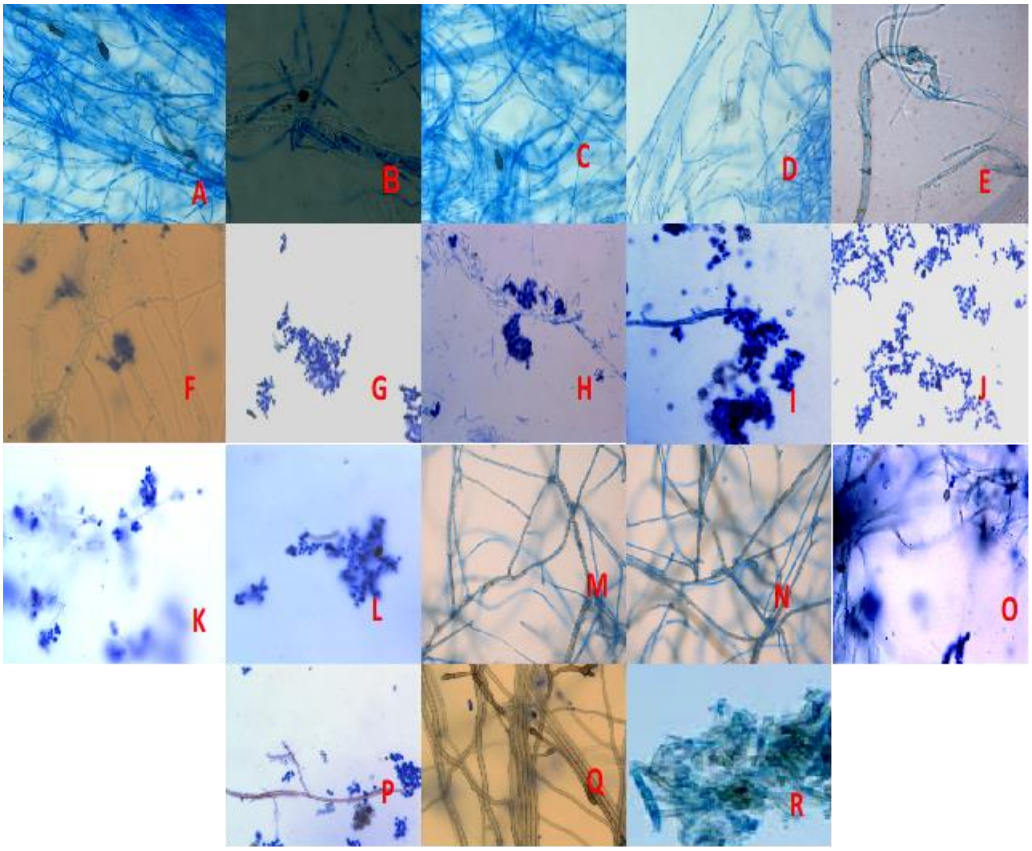

Figure 2: Morphology of 18 endophytic fungi isolated from different cultivars of *Piper nigrum* stained with lacto phenol cotton blue. A – I – PF1 – PF9 (Karimunda); J – M – PF10 - PF14 (Narayakodi); N – R – PF15 - PF18 (Kottanadan)

Molecular identification of the isolates was done based on Internal Transcribed Spacer (ITS) sequence similarity based method (**Fig.3 - 4**). The ITS sequences of the isolates were used for identification based on similarity analysis using NCBI BLAST. BLAST analysis revealed the isolates PF2, PF4, PF9 and PF13 to be 100% identical to *Phomopsis* sp. . The isolates PF10 and PF11 were 98% and 99% identical with *Mycosporella* sp. and *Pseudocercospora schizolobii*. At the same time, PF3, PF6, PF8 and PF12 were found to have 100% identity with *Colletotrichum gloeosporioides* and PF1, PF5, PF7, PF14, PF15 and PF18 showed its maximum identity of 99% with *Diaporthe* sp. respectively. The isolates PF16 and PF17 showed its maximum of 99% identity with *Pleosporales* sp. and *Fusarium* sp. (**Table 3).**

Among the various isolates obtained, PF2 (*Phomopsis* sp.), PF12 (*Colletotrichum* sp.), PF10 (*Mycosphaerella* sp.), PF7 (*Diaporthe* sp.), PF11 (*Pseudocercospora* sp.), PF17 (*Fusarium* sp.) and PF16 (*Pleosporales* sp.) which were found to be distinct in identity were selected for further studies. The results has also suggested that the members of *Colletotrichum* sp., *Phomopsis* sp. and *Diaporthe* sp. as predominant endophytic fungi in *P. nigrum*. The sequence data obtained from the study were submitted in NCBI GenBank and accession number obtained is shown in **Table 4**. The phylogenetic analysis also confirmed the distinct clustering of the isolates obtained in the study (**Fig.5**).

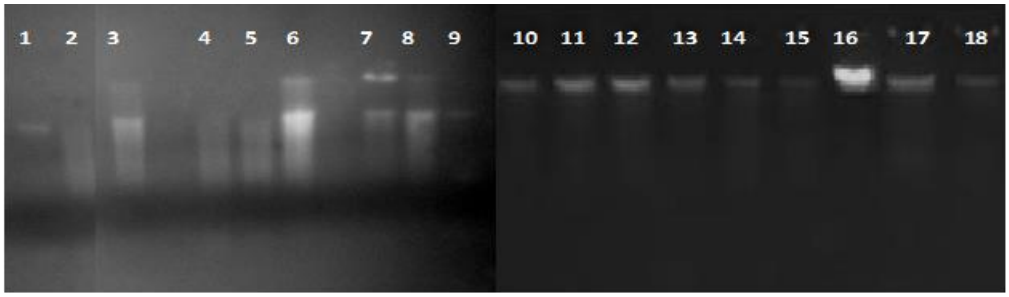

Figure 3: Genomic DNA isolated from 18 endophytic fungi from *Piper nigrum*.1 – 9 – PF1 – PF9 (Karimunda); 10 – 14 – PF10 - PF14 (Narayakodi); 15 – 18 – PF15 - PF18 (Kottanadan)

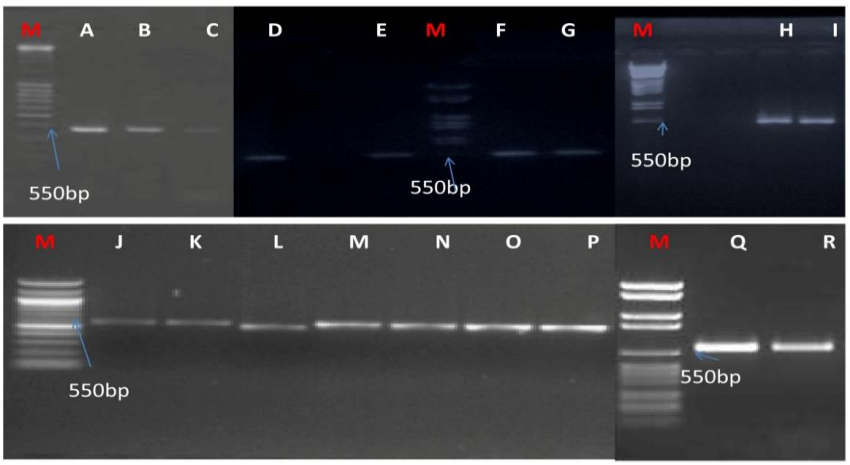

Figure 4: PCR amplification of ITS sequence from 18 endophytic fungi from *Piper nigrum* A – I – PF1 – PF9 (Karimunda); J – M – PF10 - PF14 (Narayakodi); N – R – PF15 - PF18 (Kottanadan)

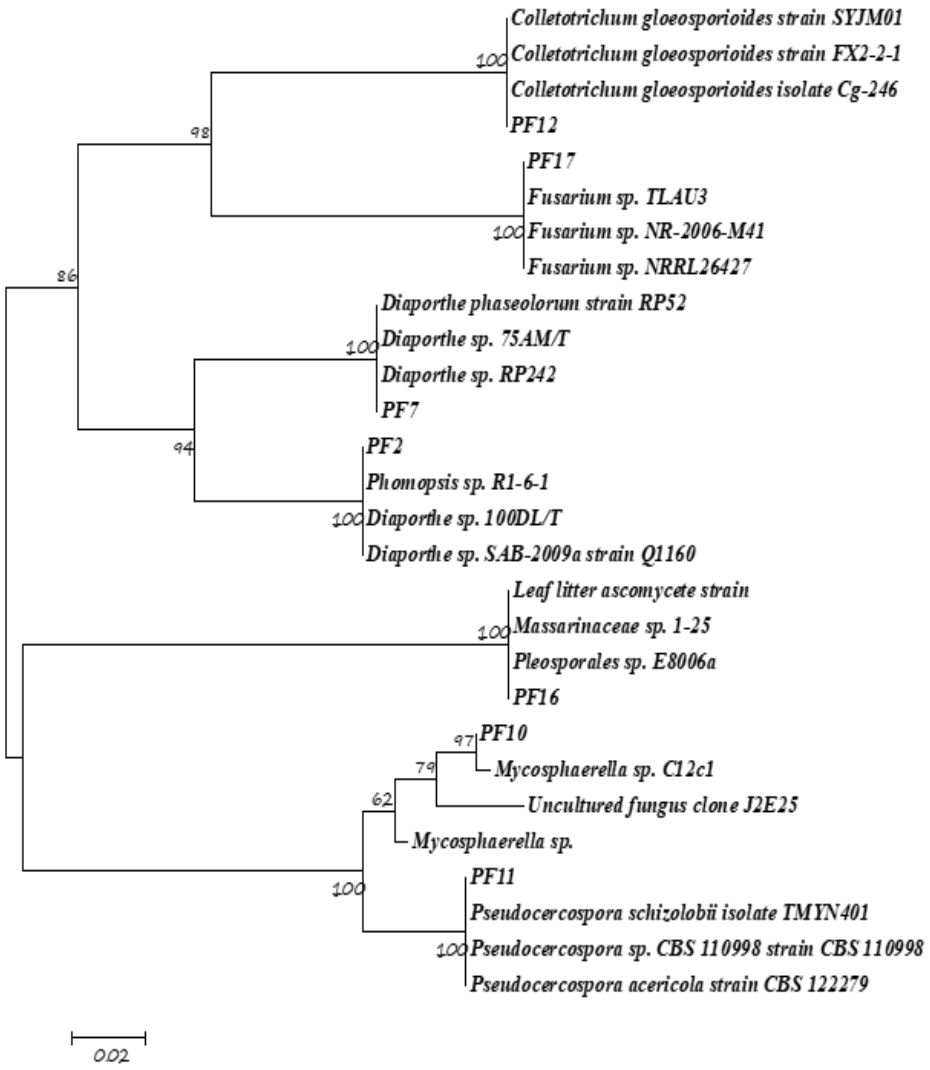

Figure 5: Phylogenetic analysis of ITS sequences of endophytic fungi isolated from *Piper nigrum*

Table 3: Summary of BLAST result of endophytic fungi isolated from *Piper nigrum.*

Organism number	Closely related species	Accession number	Identity	Organism type
PF1	*Diaporthe* sp.	JF 441198.1	99%	Endophyte (Zhong *et al.*, 2008)
PF2	*Phomopsis* sp. *R1 - 6 - 1*	HM042310.1	100%	Endophyte (Murali *et al*., 2006)
PF3	*Colletotrichum gloeosporioides Cg 246*	HQ264183.1	100%	Endophyte/ Phytopathogen (Pillai *et al*., 2015)
PF4	*Diaporthe* sp.	JF 441198.1	99%	Endophyte (Zhong *et al.*, 2008)
PF5	*Phomopsis* sp. *R1 - 6 - 1*	HM042310.1	100%	Endophyte (Murali *et al*., 2006)
PF6	*Colletotrichum gloeosporioides Cg 246*	HQ264183.1	100%	Endophyte/ Phytopathogen (Pillai *et al*., 2015)
PF7	*Diaporthe* sp.	JF 441198.1	99%	Endophyte (Zhong *et al.*, 2008)
PF8	*Colletotrichum gloeosporioides Cg 246*	HQ264183.1	100%	Endophyte/ Phytopathogen (Pillai *et al*., 2015)
PF9	*Phomopsis* sp. *R1 - 6 - 1*	HM042310.1	100%	Endophyte (Murali *et al*., 2006)
PF10	*Mycosporella* sp. *C12c1*	JX436786.1	99%	Endophyte (Moreno *et al.*, 2011)
PF11	*Pseudocercospora* sp.	JQ676195.1	99%	Endophyte (De Abreu *et al*., 2010)
PF12	*Colletotrichum gloeosporioides Cg 246*	HQ264183.1	100%	Endophyte/ Phytopathogen (Pillai *et al*., 2015)
PF13	*Phomopsis* sp. *R1 - 6 - 1*	HM042310.1	100%	Endophyte (Murali *et al*., 2006)
PF14	*Diaporthe* sp.	JF 441198.1	99%	Endophyte (Zhong *et al.*, 2008)
PF15	*Diaporthe* sp.	JF 441198.1	99%	Endophyte (Zhong *et al.*, 2008)
PF16	*Pleosporales* sp.	HQ008911.1	99%	Endophyte (Luiz *et al*., 2012)
PF17	*Fusarium* sp.	EU 352873.1	99%	Endophyte (Latiffah and Chua, 2013)
PF18	*Diaporthe* sp.	JF 441198.1	99%	Endophyte (Zhong *et al.*, 2008)

Table 4: NCBI GenBank accession number of the endophytic fungi

Endophytic Fungi	Name of Organism	NCBI Accession Number
PF2	*Phomopsis* sp.	KC213980
PF7	*Diaporthe* sp.	KC213981
PF10	*Mycosporella* sp.	KC213982
PF11	*Pseudocercospora* sp.	KC213983
PF12	*Colletotrichum gloeosporioides*	KC213984
PF16	*Pleosporales* sp.	KC213985
PF17	*Fusarium* sp.	KC213986

4. Discussion

Endophytic fungi are one of the most unexplored groups of organisms in terms of its biosynthetic potential (Wen et al., 2023). Endophytic microorganisms have the potential even to produce compounds that are similar to that of their host plants. So the endophytic fungi from medicinal plants are very important (Akram et al., 2023).

Plants of *Taxus* sp. was the only source of taxol production before the demonstration of *Taxomyces andreanae* for taxol production. Fungal production of taxol has also been mimicked an endophytic fungi *P. microspora* associated with *T. wallacbiana (*Strobel *et al.,* 1996). Now many endophytic fungi are known to have the potential to produce taxol, which itself is representation of enormous biosynthetic potential of endophytic fungi. This also confirms endophytic fungi from medicinal plants as untapped source for drug discovery. Since *Piper nigrum* is having various medicinal properties, endophytic fungi associated with them can have much application (Hashem et al., 2023).

The endophytic fungi obtained from the stem of *P. nigrum* was identified by sequencing the ITS region. The results, based on sequence analysis using NCBI BLAST revealed the presence of seven different species of endophytic fungi like *Colletotrichum* sp., *Diaporthe* sp., *Phomopsis* sp., *Mycosporella* sp., *Fusarium* sp., *Pleosporales* sp. and *Pseudocercospora schizolobii* were isolated from *Piper nigrum*.

Even though much more species of endophytic fungi can be expected from the plant, the conditions and media used in the current study might have favored the growth of the species obtained. Among these isolates, *Colletotrichum* sp. has been reported as common endophytes of *Taxus mairei* and other plants (Adhikari *et al*., 2023). Identification of species of *Colletotrichum* sp. and *Phomopsis* sp. as endophyte of *Piper hispidum* which belongs to the same family of the plant selected for the study is supportive to the results obtained in the study (Ravely *et al*., 2015). Very interestingly, *Colletotrichum gloeosporioides* associated endophytically with *Justicisa gendarussa* was shown to have the ability to produce not only taxol but also industrially important enzymes like α amylase and glucoamylase (Yehia., 2023).

The genus *Phomopsis* contains potential group of fungi with the ability to produce diverse secondary metabolites including xanthones, phomosines, cyclochalisines, convolvulanic acids and mycotoxins such as phompopsin A (Xu *et al*., 2021). These compounds are known to have a significant anti malarial, anti tubercular and anti cancer activity. Bioactive metabolites like cycloepoxylactone and phomolactonea A - C are reported to be present in the culture extract of endophytic fungus *Phomopsis* (Isaka *et al*., 2001). Members of *Diaporthe* sp. have the ability to produce

an array of compounds with potential application as biocontrol agents, plant growth enhancers and anti microbial agents (Mishra et al., 2011). An endophytic species of *Diaporthe* isolated from mangrove was found to have the ability to synthesise 3 - hydroxypropionic acid (3 - HPA) which showed specific anti microbial activities against both *Staphylococcus aureus* and *Salmonella typhimurium (*Sebastians *et al*., 2012). Endophytic *Diaporthe* sp. isolated from the rhizome of *Curcuma longa* was reported to have the capability to produce novel compounds like neohexahydrocurcumin which is a colourless derivative formed by the biotransformation of curcumin (Maehara *et al*., 2011)

Several species of endophytic *Fusarium* are known to have the ability to produce metabolites with anti microbial and anticancer activity. Endophytic fungal strains of *Fusarium solani* isolated from *Apodytes dimidiata* were demonstrated to produce different derivatives of camptothecin (Shweta *et al*., 2010). Also *Fusarium* sp. isolated as endophyte from Himalayan yew was shown to have potent anti microbial activity against clinically significant micro organisms (Tayung *et al*., 2011). The strains of *Fusarium* sp. endophytically associated with *Rhizophora annamalayana* and *Taxus celebica* were reported for its capability to produce potent anticancer compounds like taxol and paclitaxel respectively (Alaganadham *et al*., 2012). *Pleosporales* sp. has been reported both as endophyte and epiphyte from a wide range of plants including mangrove plants (Zhang *et al*., 2009). The biosynthetic properties of endophytes from the genus that obtained in the study as explained above clearly indicate the isolated strains to have promising bioactive potential.

5. Conclusion

18 endophytic fungi were isolated from various cultivars (Karimunda, Narayakodi, Kottanadu) of *Piper nigrum*. Based on morphological and molecular identification, seven different endophytes were identified as *Colletotrichum* sp., *Diaporthe* sp., *Phomopsis* sp., *Mycosporella* sp., *Fusarium* sp., *Pleosporales* sp. and *Pseudocercospora* sp. *Piper nigrum* is well known for its metabolite richness and medicinal property. So the endophytic fungi isolated from *Piper nigrum* can expect to have promising bioactive metabolites.

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