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Exploring the Diversity of Culturable Foliar Endophytic Fungi in Two Cultivated Ferns: Marsilea quadrifolia, (Marsileaceae) and *Nephrolepis cordifolia* (Nephrolepidaceae)

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ABSTRACT

Fungal endophytes, integral constituents of the plant microbiome, are characterized by their asymptomatic colonization of plants. While certain fungal groups like Arbuscular Mycorrhizal Fungi (AMF) and Dark Septate Endophytes (DSE) have been extensively researched in relation to pteridophytes, investigations in this domain remain limited. In this study, we delved into the presence of endophytic fungi within two fern species: Marsilea quadrifolia L., an aquatic fern from the Marsileaceae family, and Nephrolepis cordifolia (L.) C.Presl, a terrestrial fern belonging to the Nephrolepidaceae family. From a total of 200 plant segments examined, a remarkable 160 sporulating fungal isolates were successfully cultured. Of the remaining 40 isolates, no growth was observed in 26 plates, while in 14 plates non-sporulating isolates were frequently growing and were treated as Mycelia sterilia. The culmination of our investigation yielded a rich fungal diversity, encompassing representatives from 11 genera belonging to nine families. This comprehensive study underscores the importance of exploring fungal endophytes in pteridophytes, shedding light on the intricate relationships between these fungi and their host plants within the Marsilea and Nephrolepis species. Such insights are crucial for advancing our understanding of plant-fungal interactions in diverse ecological niches and may have broader implications for plant health and ecosystem dynamics.

Keywords: Culture dependent, Endophytes, Rajasthan, Statistical Analysis

INTRODUCTION

Endophytes constitute a fascinating category of endosymbiotic microorganisms, encompassing bacteria, fungi, archaea, and protists, which reside within the inter and intracellular spaces of plants (Liarzi and Ezra, 2014; Gouda *et al.*, 2016). In this mutually beneficial relationship, plants offer endophytes a hospitable habitat and sustenance, while endophytes reciprocate by safeguarding plants from diseases, pests, and environmental stresses such as salinity and drought (Bacon and White, 2016).

Endophytes have been discovered across diverse plant groups (Rashmi *et al*., 2019), including the enigmatic realm of pteridophytes and ferns (Petrini, 1986; Raviraja *et al*. 1996; Sati and Belwal, 2005; Sati *et al*., 2009; Kumaresan *et al*., 2013; Udaya Prakash *et al*., 2018). Within the domain of pteridophytes, substantial attention has been directed towards the exploration of foliar endophytic fungi in various taxa of *Marsilea* and *Nephrolepis* ferns. Though previous studies have delved into the intricacies of endophytic associations in species such as *Marsilea minuta* (UdayaPrakash *et al*., 2018), *Nephrolepis biserrata* (Olmo-Ruiz and Arnold, 2014), *N. undulata* (Olmo-Ruiz and Arnold, 2017), *N. cordifolia*, and *Nephrolepis* sp. (Seifollahi *et al*., 2023). However, this study marks the first investigation into the foliar endophytes of *M. quadrifolia* across the globe. Additionally, *N. cordifolia* was selected to assess any deviations in foliar endophyte diversity compared to previous studies and also to compare diversity between these two genera, given their differing leaf shapes and sizes.

MATERIALS AND METHODS

Sample Collection: Healthy leaves of the above mentioned ferns, were taken from the plants growing in Department of Botany at the University of Rajasthan, Jaipur (26°89'00.8"N 75°81'77.23"E). 25 to 30 pinnule per plant were carefully selected for their pristine condition and transported to the laboratory for further analysis. **Isolation of Endophytes**: The isolation and subsequent identification of endophytic fungal strains followed a meticulously executed procedure, adapted from the modified surface sterilization method described by Tripathi and Joshi (2019). Here is a detailed breakdown of the isolation process:

i) Sample Preparation: Healthy pteridophyte leaves of ca. 2 cm^2 were initially cleaned using tap water to remove any surface impurities or contaminants.

ii) Surface Sterilization: The cleaned leaf samples underwent a rigorous surface sterilization process to eliminate external microorganisms. Sequential immersion steps were employed, consisting of a 10-second immersion in 50% ethanol, followed by a 30-second immersion in 0.5% sodium hypochlorite (NaOCl). Subsequently, the sterilized leaf samples were thoroughly rinsed in sterile double-distilled water for 30 seconds to remove any residual chemicals.

iii) Drying and Segmentation: After surface sterilization, the leaf samples were carefully air-dried under aseptic conditions. Once completely dry, the samples were delicately cut into small pieces, each measuring 0.5 cm^2.

iv) Culturing on Potato Dextrose Agar (PDA): The surface-sterilized leaf segments were placed onto Petri dishes (one segment per plate) containing potato dextrose agar (PDA) and a total of 200 plates (i.e. 100 per fern) were inoculated with leaf segments. These PDA plates were supplemented with 150 mg/l streptomycin to inhibit bacterial growth. The Petri dishes were securely sealed using Parafilm™ to prevent contamination.

v) Sterilization Efficacy Testing: To verify the effectiveness of the surface sterilization process, a portion (500 µl) of the final rinsing water from the sterilization procedure was plated onto fresh PDA plates. Additionally, tissue imprints from the leaf segments were made on fresh PDA plates. These plates were then incubated at a controlled temperature of 26 ± 1°C until fungal growth became evident.

vi) Sub-Culturing and Pure Culture Isolation: Once fungal growth was observed, the tips of the emerging fungal mycelia were carefully excised and transferred to new PDA plates to establish pure cultures. Periodic examinations of these pure cultures were conducted to monitor their growth and development.

vii) Slide Preparation and Identification: After 14-days incubation, the endophytic fungi that successfully grew on the culture media were further subjected to morphological identification. Slides containing pure cultures were prepared using the scotch tape technique. These prepared slides were then subjected to identification using established identification keys (Barnett & Hunter 1986, Dou et al. 2014). For those fungal isolates that did not produce spores (Mycelia sterilia), their characteristics were documented.

viii) Documentation and Depository: The emergent fungal isolates were thoroughly documented and photographed. Living vouchers of these fungi were deposited in the herbarium of the Department of Botany at the University of Rajasthan, Jaipur, India, under the acronym RUBL.

Statistical Analysis and Species Diversity Indices: In order to comprehensively assess the endophytic fungal communities within *M. quadrifolia* and *N. cordifolia*, following statistical parameters and species diversity indices were employed:

Colonization Frequency: This metric provides insight into the prevalence of endophytic fungal colonization within the plant segments, and was calculated using the following formula (Fisher and Petrini, 1987).

$$\text{Colonization frequency} = \frac{\text{Number of segments colonized} \geq 1 \text{ isolate}}{\text{Total number of segments screened}} \text{ x } 100$$

Relative density of colonization (rD %): This parameter offers an assessment of the abundance of individual fungal species within the sampled segments and was computed using the following formula (Fisher and Petrini, 1987):

$$\text{Relative density of colonization} = \frac{\text{Total number of individuals of a fungi recorded}}{\text{Total number of segments screened}} \text{ x } 100$$

Jaccard's Similarity Coefficient: To compare the endophytic assemblages between different host colonizing different habitats (aquatic and terrestrial), the Jaccard's Similarity Coefficient was employed (Arnold *et al*., 2000), and was determined by the formula:

$$\text{Similarity coefficient} = \frac{C}{A+B-C}$$

where 'A' and 'B' denote the total number of fungal species isolated from different habitats, while 'C' represents the number of fungal species shared in common between them.

RESULTS AND DISCUSSION

In the present study, a thorough investigation of endophytic fungal communities within *M. quadrifolia* and *N. cordifolia* ferns was undertaken, yielding valuable insights into their diversity, colonization frequencies, and relative densities.

A total of 160 sporulating isolates (85 from *N. cordifolia* and 75 from *M. quadrifolia*) were successfully isolated from 200 segments of both fern species. Of the remaining 40 isolates, no growth was observed in 26 plates, while in 14 plates non-sporulating isolates were frequently growing and were treated as *Mycelia sterilia* based on fungal culture morphology and spore characteristics. This exhaustive effort resulted in the identification of a diverse array of endophytic fungi, encompassing 9 families belonging to 11 genera, and a total of 17 distinct fungal taxa (**Figure 1 & 2**).

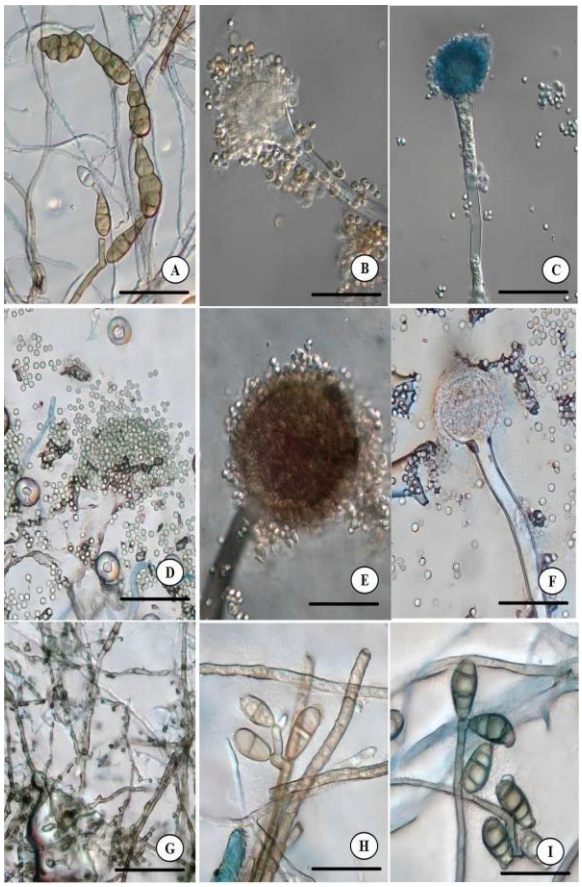


Figure 1: Foliar endophytic fungi isolated from *Marsilea quadrifolia* and *Nephrolepis cordifolia*. **A**, *Alternaria* sp.; **B**, *Aspergillus flavus*; **C**, *Aspergillus fumigatus*; **D**, *Aspergillus nidulans*; **E**, *Aspergillus niger*; **F**, *Aspergillus* sp.; **G**, *Aureobasidium* sp.; **H**, *Bipolaris* sp. 1; **I**, *Bipolaris* sp. 2 (Scale bar: A to H = 10 µm).

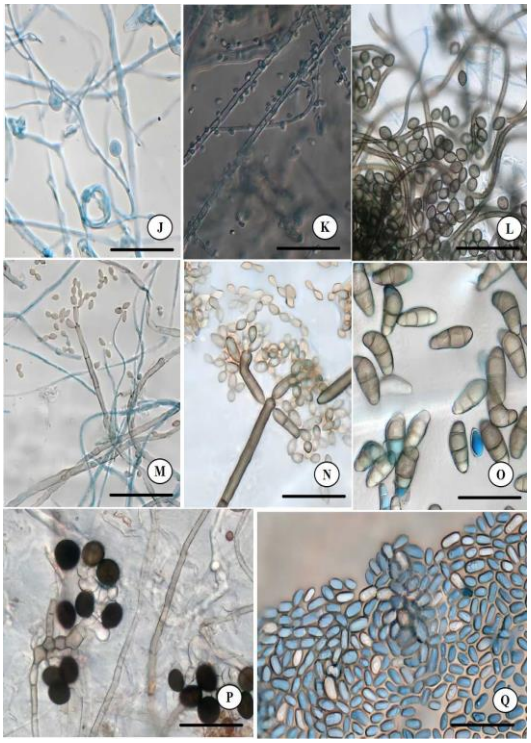


Figure 2: J, *Blastomyces* sp.; **K**, *Candida* sp.; **L**, *Chaetomium globosum*; **M**, *Cladosporium* sp.; **N**, *Cladosporium tenuissimum*; **O**, *Curvularia* sp.; **P**, *Nigrospora oryzae*; **Q**, *Phoma* sp. (Scale bar: J to Q = 10 µm).

Nephrolepis cordifolia exhibited a notably high colonization frequency of 91%, surpassing the 83% colonization frequency observed in *Marsilea quadrifolia*. This indicates a relatively higher degree of colonization in *N. cordifolia*. Additionally, this frequency encompasses non-sporulating isolates categorized under *Mycelia sterilia* (06 taxa), as depicted.. This discrepancy may stem from the ecological differences between the two species. *Nephrolepis*, being a terrestrial plant, naturally encounters a greater propensity for fungal colonization in soil environments compared to *Marsilea*, which predominantly inhabits aquatic habitats. The variance in habitat preferences likely influences the interactions between these plants and fungi. Terrestrial environments provide ample opportunities for fungal colonization, as soil offers a rich and diverse substrate for fungal growth and establishment. In contrast, the aquatic habitat of *Marsilea* may present challenges for fungal colonization due to the distinct physical and chemical properties of water-based ecosystems. Consequently, the observed differences in colonization rates between *Nephrolepis* and *Marsilea* highlight the intricate interplay between plant ecology and fungal symbiosis. Understanding

these ecological nuances is essential for elucidating the dynamics of plant-fungal interactions and their implications for ecosystem functioning.

For *M. quadrifolia*, the following 08 foliar endophytes were identified for the first time in this fern species: *Alternaria* sp., *Aspergillus fumigatus*, *Aspergillus nidulans*, *Bipolaris* sp. 1, *Blastomyces* sp., *Candida* sp., *Cladosporium* sp. and *Cladosporium tenuissimum*. This discovery adds to our understanding of the microbial diversity within *M. quadrifolia* and underscores the importance of exploring previously uncharted microbial associations.

Similarly, our investigation of *N. cordifolia* revealed a unique set of foliar endophytes that had not been previously reported in connection with this fern species (Seifollahi *et al*. 2023). These include *Alternaria* sp., *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus* sp., *Aureobasidium* sp., *Bipolaris* sp. 2, *Chaetomium globosum*, *Curvularia* sp., *Nigrospora oryzae*, and *Phoma* sp. The isolation of these endophytes from *N. cordifolia* expands our knowledge of the plants associated microbial community (**Table 1**).

Table 1: Diversity of foliar endophytic fungi associated with species of *Marsilea* and *Nephrolepis* in the present and previous studies.

S. No.	Endophytic fungi	*M. minuta* (Udaya Prakash *et al.* 2018)	*M. quadrifolia* (present paper)	*N. biserrata* (Olmo-Ruiz & Arnold 2014)	*N. cordifolia* (Seifollahi *et al.* 2023)	*N. cordifolia* (present paper)	*Nephrolepis* sp. (Seifollahi *et al.* 2023)	*N. undulata* (Olmo-Ruiz & Arnold 2017)
1.	*Acremonium* sp.	+	-	-	-	-	-	-
2.	*Alternaria alternata* (Fr.) Keissl.	+	-	-	-	-	-	-
3.	***Alternaria* sp.**	-	+	-	-	+	-	-
4.	*Annulohypoxylon* sp.	-	-	+	-	-	-	-
5.	***Aspergillus flavus* Link**	-	-	-	-	+	-	-
6.	***Aspergillus fumigatus* Fresen.**	-	+	-	-	+	-	-
7.	***Aspergillus nidulans* (Eidam) G. Winter**	-	+	-	-	+	-	-
8.	***Aspergillus niger* Tieghem**	-	-	-	-	+	-	-
9.	***Aspergillus* sp.**	-	-	-	-	+	-	-
10.	*Aureobasidium pullulans* (de Bary & Löwenthal) G. Arnaud	-	-	-	-	-	-	-
11.	***Aureobasidium* sp.**	-	-	-	-	+	-	-
12.	*Biatriospora* sp.	-	-	-	-	-	-	+
13.	***Bipolaris* sp. 1**	-	+	-	-	±	-	-
14.	***Bipolaris* sp. 2**	-	-	-	-	+	-	-
15.	***Blastomyces* sp.**	-	+	-	-	-	-	-
16.	*Botryodiplodia thoebramae* Pat	+	-	-	-	-	-	-
17.	***Candida* sp.**	-	+	-	-	-	-	-
18.	***Chaetomium globosum* Kunze ex Fries**	-	-	-	-	+	-	-
19.	***Cladosporium* sp.**	-	+	-	-	-	-	-
20.	***Cladosporium tenuissimum* Cooke**	-	+	-	-	-	-	-
21.	*Colletotrichum fructiola* Prihastuti, L. Cai & K.D. Hyde	-	-	-	+	-	-	-

22.	*Colletotrichum gigasporum* E.F. Rakotoniriana & Munaut	-	-	-	+	-	-	-
23.	*Colletotrichum pandanicola* Tibpromma & K.D. Hyde	-	-	-	+	-	-	-
24.	*Colletotrichum polypodialium* Seifollahli, Jayawardena & K.D. Hyde	-	-	-	-	-	+	-
25.	*Colletotrichum* sp.	+	-	-	-	-	-	-
26.	*Curvularia lunata* (Wakker) Boedijn	+	-	-	-	-	-	-
27.	***Curvularia* sp.**	-	-	-	-	+	-	-
28.	*Diaporthe heveae* Petch	-	-	-	+	-	-	-
29.	*Diaporthe tectonendophytica* Doilom, A.J. Dissanayake& K.D. Hyde	-	-	-	+	-	-	-
30.	*Drechslera australiensis* (M.B. Ellis) Manamgoda, L.Cai. & K.D. Hyde	+	-	-	-	-	-	-
31.	*Drechslera halodes* (Drechsler) Subram. & B.L. Jain	+	-	-	-	-	-	-
32.	*Fusarium* sp.	+	-	-	-	-	-	-
33.	*Lasiodiplodia thailandica* T. Trakunyingcharoen, L. Lombard & Crous	-	-	-	+	-	-	-
34.	*Neopestalotiopsis guajavicola* I.U. Haq, S. Ijaz & N.A. Khan	-	-	-	-	-	+	-
35.	*Neopestalotiopsis psidii* I.U. Haq, S. Ijaz & N.A. Khan	-	-	-	+	-	-	-
36.	***Nigrospora oryzae* (Berk. & Broome) Petch**	-	-	-	-	+	-	-
37.	*Penicillium* sp.	+	-	-	-	-	-	-
38.	*Pestalotiopsis dracontomelon* Maharachch. & K.D. Hyde	-	-	-	+	-	-	-
39.	***Phoma* sp.**	+	-	-	-	+	-	-
40.	*Talaromyces funiculosus* (Thom) Samson, Yilmaz, Frisvad & Seifer	+	-	-	-	-	-	-
	Total	11	08	01	08	13	02	01

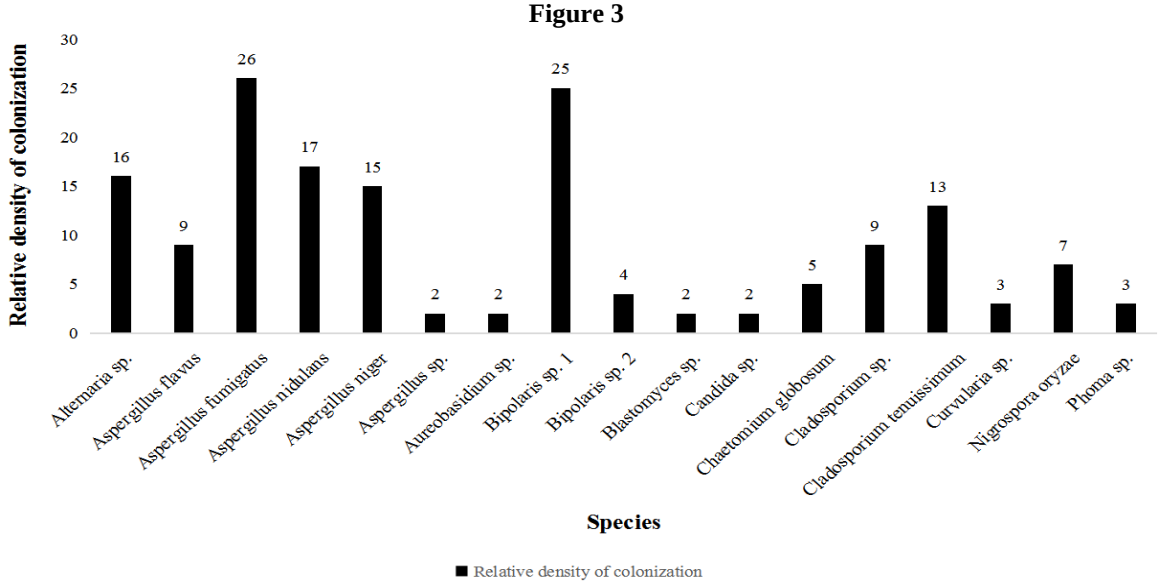
Note: species in bold are isolated for the first time

The relative density of colonization revealed that *Aspergillus fumigatus* held dominance at 16.25%, followed closely by *Bipolaris* sp 1 (15.62%), *Aspergillus nidulans* (10.62%), *Alternaria* sp. (10%), *Aspergillus niger* (9.37%), *Cladosporium tenuissimum* (8.12%), *Cladosporium* sp. (5.62%), *Aspergillus flavus* (5.62%), *Nigrospora oryzae* (4.37%), *Chaetomium globosum* (3.12%), *Bipolaris* sp. 2 (2.5%), *Phoma* sp (1.87%), *Curvularia* sp.

(1.87%), *Candida* sp. (1.25%), *Blastomyces* sp. (1.25%), and *Aureobasidium* sp. (1.25%) (**Figure 3**).

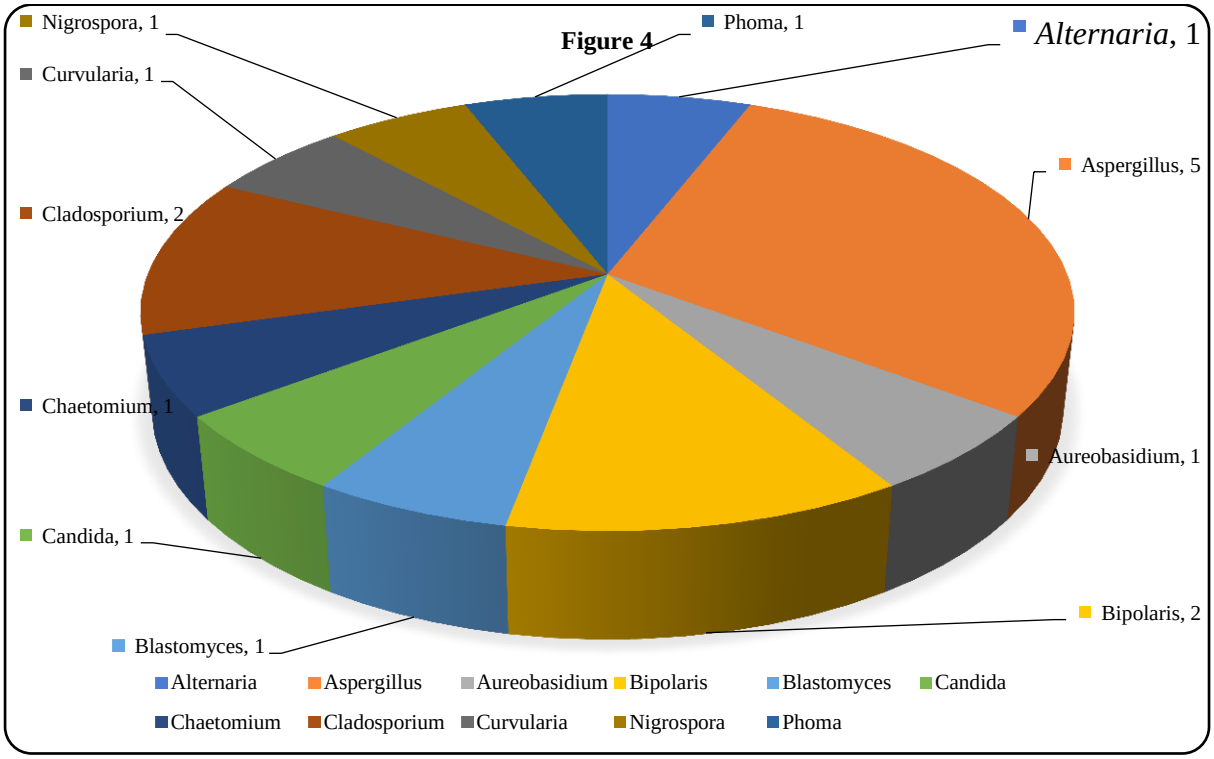
Furthermore, the relative density of the foliar endophyte *Alternaria* sp. (ranging from 8.23% to 12%) and *Aspergillus fumigatus* (ranging from 11.76% to 21.33%) displayed variations in relative abundance when transitioning from *Nephrolepis cordifolia* to *Marsilea quadrifolia*.

Figure 3



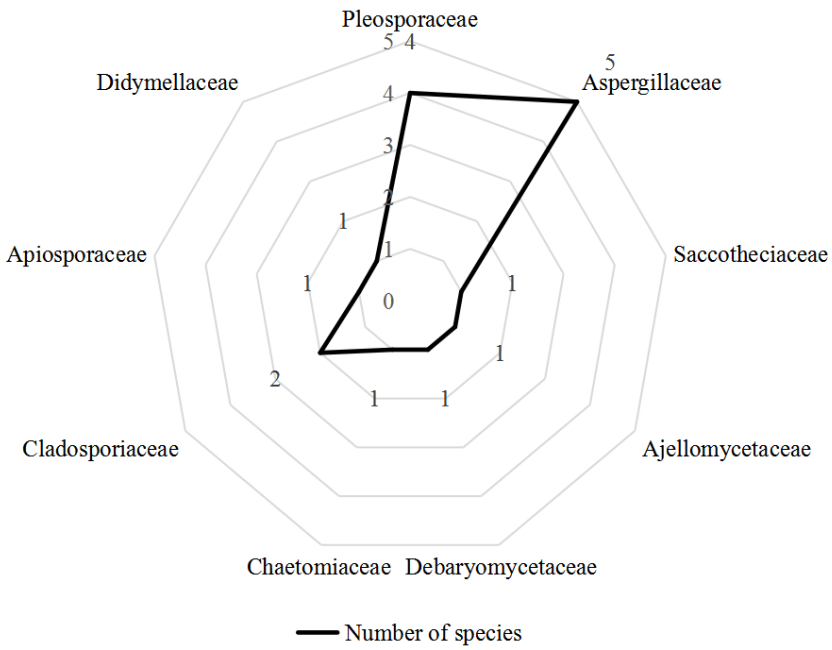
As for genera, *Aspergillus* dominated at 26.41%, followed by *Bipolaris* (11.76%), *Cladosporium* (11.76%), *Alternaria* (5.88%), *Aureobasidium* (5.88%), *Blastomyces* (5.88%), *Candida* (5.88%), *Chaetomium* (5.88%), *Curvularia* (5.88%), and *Nigrospora* (5.88%) (**Figure 4**).

Figure 4



The family Aspergillaceae (29.40%) and the order Eurotiales (29.40%) were particularly dominant, followed by Pleosporaceae (23.50%), Cladosporiaceae (11.7%), Ajellomycetaceae (5.8%), Apiosporaceae (5.8%), Chaetomiaceae (5.8%), Debaryomycetaceae (5.8%), Didymellaceae (5.8%), and Saccotheciaceae (5.8%) (**Figure 5**).

Figure 5



Class-wise, Dothideomycetes exhibited dominance at 47.0%, followed by Eurotiomycetes (35.20%), Sordariomycetes (11.7%), and Saccharomycetes (5.8%).

Jaccard's Species Diversity index revealed that both fern species shared four common fungal species, excluding *Mycelia sterilia* (**Table 2**).

Table 2: Jaccard's Similarity index between isolated fungal species from *Marsilea quadrifolia* (M) and *Nephrolepis cordifolia* (N)

	***Alternaria* sp. M**	***Alternaria* sp. N**	***Aspergillus fumigatus* M**	***Aspergillus fumigatus* N**	***Aspergillus nidulans* M**	***Aspergillus nidulans* N**	***Bipolaris* sp. 1 M**	***Bipolaris* sp. 1 N**
***Alternaria* sp. M**	1							
***Alternaria* sp. N**	0.77	1						
***Aspergillus fumigatus* M**	0	0	1					
***Aspergillus fumigatus* N**	0	0	0.62	1				
***Aspergillus nidulans* M**	0	0	0	0	1			
***Aspergillus nidulans* N**	0	0	0	0	0.13	1		
***Bipolaris* sp. 1 M**	0	0	0	0	0	0	1	
***Bipolaris* sp. 1 N**	0	0	0	0	0	0	0.56	1

CONCLUSION

This study contributes to the limited body of research comparing foliar fungal endophytic communities in aquatic and terrestrial fern species. Among the 17 identified fungal species (excluding Mycelia sterilia), only four were common to both aquatic and terrestrial ferns. The similarity index between populations allowed for a direct comparison of species diversity between *M. quadrifolia* and *N. cordifolia*. Statistical analyses unveiled distinct levels of endophytic community diversity between different fern species, likely influenced by habitat-dependent factors. These

findings provide valuable insights into the diversity and distribution of endophytic fungal communities in aquatic and terrestrial ferns, shedding light on the intricate relationships between fern hosts and their associated fungal symbionts. However, the primary limitation of the culture-dependent approach is its inability to isolate unculturable species, along with some slow-growing or weakly competitive ones. Additionally, taxonomic resolution often remains incomplete, with many taxa not identified to the species level. To address these limitations, the culture-independent approach has emerged as a promising alternative. Yet, it's worth noting that studies have revealed discrepancies between endophytic fungi recovered through culture-dependent and culture-independent methods. Some strains isolated through the former have not been detected via the latter, suggesting that each method captures a unique subset of the fungal community (Pancher et al. 2012, Kraková et al. 2017, Mendoza et al. 2017). Consequently, to obtain a more comprehensive understanding of endophytic fungal diversity, it's essential to integrate both approaches. By combining the strengths of each method, researchers can better estimate within-community diversity and gain a more holistic view of the fungal ecosystem.

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