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Comparison of colonization, diversity, and molecular phylogeny of endophytic fungi in selected traditional and newly improved rice (*Oryza sativa* L.) varieties in Sri Lanka

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Abstract

Rice (*Oryza sativa* L.) is the staple diet amongst almost all ethnic groups in Sri Lanka. The use of endophytic microorganisms inhabiting the rice plant is a promising strategy for improving paddy yields. However, little is known about the diversity and the evolutionary relationships among the species of endophytic fungal assemblages associated with different rice varieties in Sri Lanka. Therefore, this study was aimed at the assessment of colonization, diversity, identification, and the phylogenetic relationships of endophytic fungi present in newly improved rice varieties At 362, Bg 352, Bw 367, and a traditional variety, Suwandel. Healthy plant samples of each rice variety were collected during the two main rice-growing seasons, Maha and Yala (2019), from Anuradhapura, Kurunegala, Gampaha, and Kalutara districts in Sri Lanka. Endophytic fungi were isolated from the leaves, stems, and roots of each rice variety using previously developed protocols. All strains were categorized into thirty-nine (39) morphological groups based on colony characteristics and micro-morphological features. The Internal Transcribed Spacer (ITS) region of a representative strain of each morphological group was amplified and sequenced. They were identified by phylogenetic analysis based on sequences of ex-types. Thirty-one (31) isolates identified to species level belonged to twenty-two (22) genera within nine (9) orders. Apart from that, four (04) and three (03) isolates were identified up to generic and order levels respectively. One isolate was identified up to the family level. Out of the fungal species, seventeen (17) were novel records for Sri Lanka. Evaluation of the colonization percentages of endophytic fungal assemblages showed that the variety Suwandel collected from Kurunegala during the Maha and Yala seasons respectively had the highest colonization rate (52% and 35%). The study also showed that *Aspergillus tereus, Curvularia lunata, Dendryphiella* sp., *Fusarium falciforme, Microdochium fisheri*, and *Penicillium oxalicum* were the most dominant species in all rice varieties. Endophytic fungal diversity evaluated by Shannon's and Simpsons diversity indices showed the highest species diversity in variety Bg 352 collected from Kalutara district during the Yala season.

Keywords – Endophyte – ITS – Novel records – Poaceae

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Introduction

Endophytic fungi (EnF) are microorganisms that colonize plant tissues internally without causing apparent harm to the host and could be isolated from the plant tissues using specific growth media (Petrini & Carroll 1981, Pimentel et al. 2011, Singh & Dubey 2015). They have been reported to reduce disease incidence and enhance the growth of several economically important crops, such as banana, coffee, maize, wheat (Photita et al. 2001, Fisher et al. 1992, Cao et al. 2002, Santamaría & Bayman 2005, Köhl et al. 2015, Spagnoletti et al. 2017), and certain rice varieties (Ponnawila & Deshappriya 2014, Atugala & Deshappriya 2015, Wijesooriya & Deshappriya 2016). Endophytic fungi promote plant growth by solubilization of phosphorus, potassium, and zinc, and through the production of phytohormones (indole acetic acids, gibberellic acids, and cytokinin) (Finlay 2008, Bonfante & Genre 2010, Mathur et al. 2011, Rai et al. 2014, Mishra et al. 2015, Suman et al. 2016, Verma et al. 2017). Control of phytopathogens by EnF in their host plants is facilitated by a diverse range of mechanisms, including the production of metabolites (antibiotics, volatile compounds, and enzymes), competition for space, carbon sources, nitrogen and minerals, parasitism, and induction of systemic resistance in the plant (Vega et al. 2009, Vidal & Jaber 2015, Vega 2018, Moraga 2020). Similar effects have been evidenced with regard to some rice varieties grown in Sri Lanka (SL), where *Absidia* and *Cylindrocladium* have been shown to enhance the growth and yield of traditional rice varieties Suwandel and Kaluheenati. In addition, *Absidia*, *Acremonium*, and *Penicillium* have been shown to inhibit the rice pathogen *Magnaporthe grisea*, causing the Rice Blast disease (Atugala & Deshappriya 2015). However, more emphasis on the characterization of EnF communities of plant species of agronomic interest is needed for their effective use.

Studies on EnF have contributed to some extent toward the understanding of endophytic isolation, identification, taxonomy, diversity, and distribution, as well as biological, ecological, and physiological features (Cannon & Simmons 2002, Torres et al. 2011, Leewijit et al. 2016, Rana et al. 2019). Studies have also been conducted on systematics, evolutionary biology, mutualistic symbiosis, and EnF applications (Leuchtmann 1993, White et al. 2003, Saikkonen et al. 2004, Yuan et al. 2010). Some studies on fungal endophytes of traditional rice varieties in SL (Ponnawila & Deshappriya 2014, Atugala & Deshappriya 2015, Wijesooriya & Deshappriya 2016) have reported on the methods of isolation, growth enhancement, and disease control. However, there are no reports on EnF assemblages of newly improved rice varieties and their effects. This study focused on studying the diversity, colonization, and evolutionary relationships of EnF assemblages associated with Suwandel, a traditional rice variety, and some commonly cultivated newly improved rice varieties, At 362, Bg 352, and Bw 367.

Although many researchers have used morphological features to characterize fungal endophytes, the identification of fungal species requires more intricate characterization. The species identification of fungal endophytes has become more accurate with the recent development of advanced biomolecular techniques, such as molecular phylogeny and molecular species concepts in fungal systematics (Jiménez et al. 2010, Kaspřák et al. 2019, Irwin et al. 2021, Sun et al. 2021). Numerous type-derived sequences available in public databases, such as GenBank, have contributed immensely toward the accurate identification of fungal species. Therefore, morphological features along with molecular characteristics were used in the present study for EnF identification.

The overall aim of the present study was to study the EnF assemblages associated with selected rice varieties grown in SL, by assessing their percentage of colonization, dominance, and diversity in different geo-climatic zones of SL. Furthermore, the phylogenetic relationship of rice EnF was also studied. This study will lay a strong foundation for utilizing symbiotic associations between rice varieties and their EnF assemblages for improving rice productivity.

Materials & Methods

Sampling: ecosystems and rice varieties

Four rice varieties, Suwandel, At 362, Bg 352, and Bw 367 commonly cultivated in Sri Lanka,

were selected for this study. Ten plants of each rice variety were collected randomly from paddy fields in four different geographical locations representing dry, intermediate, and wet zones of SL, as shown in Figs 1, and 2. The climatic conditions and geographical coordinates of the study areas are shown in Table 1. Two to two and half months old healthy intact rice plants, in the early reproductive phase of growth were randomly collected from each location and immediately transported to the laboratory in labelled clean polythene bags. Samples were refrigerated at 4 ℃, and isolations were conducted within 48 hours after collection. The sampling of rice varieties was repeated during the two paddy cultivation seasons of Maha and Yala in the year 2019, since the colonization and diversity of endophytes in plants may show seasonal dimorphism.

Fig. 1 – Districts of the sites of the study. Paddy fields sampled were from Anuradhapura (dry zone), Kurunegala (intermediate zone), Gampaha, and Kalutara (wet zone).

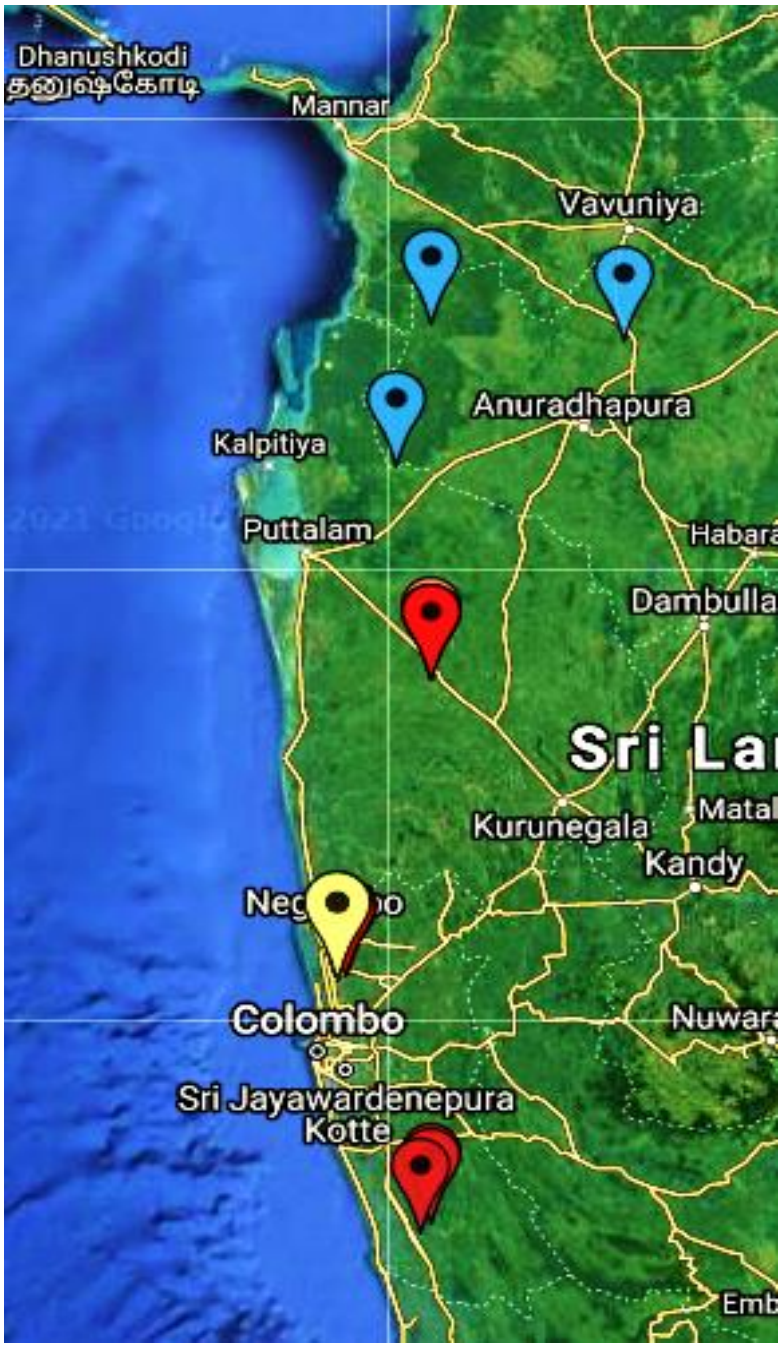


Fig. 2 – Geographical locations of the sites of the study, based on GPS mapping. The map was generated based on GPS coordinates using Mapmaker online tool (Map maker: https://maps.com).

Fungal isolation

Isolations of EnF from leaves, stems, and root segments of each rice variety collected from each selected site were done separately, according to a previously optimized surface sterilization regime (Atugala & Deshappriya 2015). Twenty pieces from each sterilized rice variety were separately transferred to a 2% (wt/vol) Malt Extract Agar (MEA) medium supplemented with Tetracycline (50 mg/L) under aseptic conditions and incubated for 10 days at room temperature. The EnF colonies emerging from the plant tissues were subcultured separately into fresh Potato Dextrose Agar (PDA) medium supplemented with Tetracycline (50 mg/L) and the plates were incubated at room temperature. Surface sterilized tissue imprints and aliquots from each final rinse were plated on MEA plates and checked for microbial growth to assess the efficacy of the surface sterilization protocol (Schulz et al. 1998).

Table 1 Sampling period and sites of collection

Season		Maha			Yala			
Geographical location	**Rice Variety**	**Period of collection**	**GPS Coordinates**	**Mean Rainfall for the month (mm)**	**Mean Temperature of the month (℃)**	**Period of collection**	**GPS Coordinates**	**Mean Rainfall for the month (mm)**	**Mean Temperature of the month (℃)**
Kurunegala Northwestern province	Bw 367	January 2019	7º 45´ 53.784" N 80º 5´ 54.6" E	0	32.12	July 2019	7º 44' 51.04032" N 80º 5' 53.5812" E	3.10	32.25
	Suwandel						7º 44' 52.81008" N 80º 5' 58.78536" E		
	Bg 352		7º 45' 53.784" N 80º 5' 54.6" E				7º 44' 52.81008" N 80º 5' 58.78536" E		
	At 362		7º 44' 51.04032" N 80º 5' 53.58084" E				7º 44' 40.09992" N 80º 6' 1.35612" E		
Kalutara Western province	Bw 367		6º 30' 53.532" N 80º 4' 32.159" E	0.102	29.8		6º 34' 35.027" N 79º 58' 24.861" E	2.25	30.25
	Suwandel		6º 32' 15.324" N 80º 5' 46.284" E				6º 34' 21.8616" N 79º 59' 58.4586" E		
	Bg 352		6º 32' 15.324" N 80º 5' 46.284" E				6º 44' 52.81008" N 79º 5' 58.78536" E		
	At 362		6º 44' 51.04032" N 80º 5' 53.58084" E				6º 44' 40.09992" N 79º 6' 1.35612" E		
Gampaha Western province	Bw 367		7º 5' 42.61873" N 79º 55' 41.2440" E	0.102	29.8		7º 6' 4.6462" N 80º 0' 58.24732" E	2.25	30.25
	Suwandel		7º 4' 45.30335" N 79º 54' 21.6301" E						
	Bg352		7º 5' 31.46035" N 79º 55' 8.43712" E				**Samples not available**		
	At 362						7º 6' 4.6462" N 80º 0' 58.24732" E		
Anuradhapura North - Central province	Bw 367	February 2019	8º 30' 0.04016" N 80º 29' 0.61866" E	3.125	31.925		8º 30' 0.04016" N 80º 29' 0.61866" E	0.48	34.6
	Suwandel		8º 13' 0.20433" N 80º 10' 0.46529" E				8º 13' 1.20433" N 80º 10' 1.46529" E		
	Bg 352		8º 32' 15.324" N 80º 5' 46.284" E				8º 30' 15.3144" N 80º 29' 26.4270" E		
	At 362								

Identification of endophytic fungal isolates

Morphological characterization of endophytic fungi

Based on colony morphology on Potato Dextrose Agar (PDA), and micro-morphological characteristics such as hyphae, conidia, and conidiophores observed using an Optika Vision upright microscope (Carl Zeiss, Germany), the isolates were grouped into thirty-nine morphological groups. Then, representative isolates from each morphological group were subjected to molecular identification.

Molecular identification of endophytic fungi

Total genomic DNA of EnF isolates selected from each morphologically different group was extracted from fresh four-day (04) old cultures grown on PDA plates. Genomic DNA extractions of EnF isolates were carried out using the DNeasy Plant Mini Kit according to the instructions given in the kit (QIAGEN/USA). The ITS region of the isolates was amplified in an automated thermal cycler (Bio-Rad T100™ Thermocycler, USA). Amplification was performed in a 25 µL reaction volume, which contained 2.5 µL of 10× PCR buffer, 1 µL of each primer, 1 µL of template DNA, and 0.25 of µL Taq DNA polymerase (Promega, Madison, WI, USA). Primers ITS1 and ITS4 (10 µM) (Glass & Donaldson 1995) were used to amplify the ITS 1, 5.8 S, and ITS 2 regions of the rDNA. The thermal cycling program described by Manamgoda et al. (2012) was followed for the amplification of the rDNA. The amplification products were separated by electrophoresis on a 2% (W/V) Agarose gel stained with Ethidium bromide (0.5 µg/mL), visualized, and photographed under 300 nm UV light. The DNA ladder of 100 bp (Promega/USA) was used as a reference. Samples with a good quality single band at the expected size range were sent to a commercial service provider, Macrogen Inc., South Korea, to perform bidirectional Sanger sequencing. The sequence data obtained for the forward and reverse strands were used to produce contigs on the sequence editing software tool Bio Edit v7.2.5. Contigs were identified using the Basic Logarithmic Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information database (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences obtained in this study were deposited in the NCBI-GenBank and the accession numbers were obtained.

Phylogenetic analysis was carried out for further identification of EnF to species level. To construct phylogenetic trees, sequences of ITS regions of all isolates were aligned with closely related ex-type and authenticated ITS sequences. Sequences were aligned using Clustal W Pairwise Sequence Alignment in Bio Edit v7.2.5. and optimized by the online sequence alignment tool MAFFT (MAFFT alignment and NJ/UPGMA phylogeny (cbrc.jp) (Katoh et al. 2009). PAUP v. 4.0b10 (Swofford 2002) was used to perform Maximum Parsimony (MP) analyses. Trees were inferred using the heuristic search option with one thousand random sequence additions. Max trees were unlimited, branches of zero length were collapsed, and all multiple equally parsimonious trees were saved. Trees were visualized with Fig Tree v1.4.2 (Page 1996) (results of individual generic trees are not shown). A Maximum Likelihood (ML) analysis was performed for all the sequences of EnF obtained from this study using the RAXML-HPC Black Box tool in the CIPRES web portal (http://www.phylo.org/portal2/) (Miller et al. 2010). The tree search included 1000 non-parametric bootstrap replicates, and the best scoring tree was generated using *Rhizopus microsporus* as an out-group (Accession No: ON063291). The RAXML bipartition tree results were visualized with Fig Tree v1.4.2 (Page 1996) and the layout was edited using Microsoft PowerPoint.

Assessment of the abundance, colonization, diversity, and dominance of endophytic fungi

The percentage of colonization (CPs) and dominance of EnF in different rice varieties collected from three climatic zones during the two cultivation seasons were separately calculated using the following formulae. The percentage abundance of each endophytic taxon isolated was also calculated (Petrini et al. 1992).

Percentage of colonization (CPs %) =

$$\frac{\text{Total no. of segments yielding } 1 \geq \text{isolates in a given trial} \times 100}{\text{Total no. of sample segments used in the same trial}}$$

Percentage dominance of a single species (%) =

$$\frac{\text{No. of isolates of each fungal species isolated} \times 100}{\text{Total number of leaf/stem/root samples in the given trial}}$$

Percentage of abundance of single genera (taxon) % =

$$\frac{\text{No. of isolates in each endophytic fungal taxon} \times 100}{\text{Total number of isolates}}$$

Diversity indices were separately evaluated for each trial using the Paleontological Statistics Software Package for Education and Data Analysis (PAST) v.4.03 (Hammer et al. 2001)

Simpson's diversity index (D) is given by

$$1 - \sum_i \left(\frac{n_i}{n}\right)^2$$

Where n_i is the number of individuals of taxon *i*, *n* is the total number of individuals, and it measures the 'evenness' of the community from 0 to 1 (Hammer et al. 2001).

Shannon-Weiner's index (H') is a diversity index, considering the number of individuals as well as the number of taxa. It varies from zero (0) for communities with only a single taxon to high values for communities with many taxa, each with few individuals (Hammer et al. 2001).

$$H = -\sum_i \frac{n_i}{n} \ln \frac{n_i}{n}$$

Results

Identification of endophytic fungi

Morphological identification

A total of five hundred and fifteen (515) fungal isolates were obtained from the rice varieties collected during the two cultivation seasons from four geographical locations. They were grouped into thirty-nine (39) morphological groups based on the colony (Fig. 3) and micro-morphological characteristics. Out of these, twenty-six (26) morphological groups were identified to their generic level based on their micro-morphological characteristics. They were classified under fourteen (14) genera as *Aspergillus*, *Bipolaris*, *Curvularia*, *Daldinia*, *Exserohilum*, *Fusarium*, *Microdochium*, *Penicillium*, *Rhizopus*, *Rhizoctonia*, *Ramichroridium*, *Sarocladium*, *Talaromyces*, and *Zopifiella* based on morphology. Thirteen (13) other morphological groups were sterile forms that did not form any spores on PDA under laboratory conditions despite various attempts (Table 2). Therefore, those EnF isolates were subjected to molecular identification.

Table 2 Taxonomic placement of sporulating and sterile morphological groups of endophytic fungi isolated in this study as inferred from BLAST searches and MP analysis.

No.	Isolate code	Nearest match in the NCBI database	Classification (Class, order)	The accession number of the representative isolate	Total No. of isolates
Sporulating endophytic fungi					
1	KuAtR8	*Aspergillus fischeri**	*Eurotiomycetes, Eurotiales*	ON063267	9
2	yKuSuR15a	*Aspergillus assituensis**	*Eurotiomycetes, Eurotiales*	ON063268	1
3	KuBgR12	*Aspergillus tereus*	*Eurotiomycetes, Eurotiales*	ON063269	21
4	*Bipolaris* sp	*Bipolaris oryzae*	*Dothideomycetes, Pleosporales*	ON063270	9
5	MSuL9	*Curvularia coicis**	*Dothideomycetes, Pleosporales*	ON063271	9
6	KuSuS7	*Curvularia chiangmaiensis**	*Dothideomycetes, Pleosporales*	ON063272	5
7	yBSuL3	*Curvularia lunata*	*Dothideomycetes, Pleosporales*	ON063273	20
8	yBSuL8	*Curvularia plantarum*	*Dothideomycetes, Pleosporales*	ON063274	23
9	yKuSuL1A	*Curvularia* sp. 1	*Dothideomycetes, Pleosporales*	ON063275	6
10	KuBgL18	*Daldinia eschscholtzii*	*Sordariomycetes, Xylariales*	ON063276	14
11	JSuL6	*Exserohilum rostratum*	*Dothideomycetes, Pleosporales*	ON063278	8
12	ABwR14	*Fusarium annulatum**	*Sordariomycetes, Hypocreales*	ON063279	13
13	yKuBgL4	*Fusarium chlamydosporum**	*Sordariomycetes, Hypocreales*	ON063280	4
14	yABwR18	*Fusarium falciforme**	*Sordariomycetes, Hypocreales*	ON063281	17
15	KuSuL6	*Microdochium fisheri**	*Sordariomycetes, Xylariales*	ON063284	51
16	MBWR4	*Penicillium oxalicum*	*Eurotiomycetes, Eurotiales*	ON063286	26
17	MBgS2	*Ramichloridium apiculatum*	*Dothideomycetes, Capnodiales*	ON063289	1
18	Rhi	*Rhizoctonia solani*	*Agaricomycetes, Cantharellales*	ON063290	9
19	yKuSuR3	*Rhizopus microsporus*	*Mucoromycetes, Mucorales*	ON063291	20
20	ASuS1	*Sarocladium oryzae*	*Sordariomycetes, Hypocreales*	ON063292	18
21	yKuAtS16	*Talaromyces veerkampii**	*Eurotiomycetes, Eurotiales*	ON063293	14
22	yASuS2	*Talaromyces funiculosus*	*Eurotiomycetes, Eurotiales*	ON063294	3
23	yBAtR19	*Talaromyces liani**	*Eurotiomycetes, Eurotiales*	ON063295	3
24	yABgR6	*Talaromyces purpureogenus*	*Eurotiomycetes, Eurotiales*	ON063296	17
25	yABwR16	*Talaromyces stipitatus**	*Eurotiomycetes, Eurotiales*	ON063297	9
26	MBwL20	*Zopfiella pilifera**	*Sordariomycetes, Sordariales*	ON063302	1
Non – sporulating endophytic fungi					
27	ASuR4	*Achroiostachys humicola**	*Sordariomycetes, Hypocreales*	ON063266	3
28	yKuSuL17A	*Dendryphiella* sp.	*Dothideomycetes, Pleosporales*	ON063277	48
29	JAtR12	*Magnaporthaceae* sp.	*Sordariomycetes, Magnaporthales*	ON063283	21

Table 2 Continued.

No.	Isolate code	Nearest match in the NCBI database	Classification (Class, order)	The accession number of the representative isolate	Total No. of isolates
30	MSuS17	*Magnaporthe salvinia*	*Sordariomycetes, Magnaporthales*	ON063282	7
31	JBgL4	*Nigrospora oryzae*	*Sordariomycetes, Xylariales*	ON063285	15
32	YABwR3	*Pseudothielavia terricola**	*Sordariomycetes, Sordariales*	ON063287	8
33	yKuBwR20	*Pyrenochaetopsis paucisetosa*	*Dothideomycetes, Pleosporales*	ON063288	10
34	ASuR1	*Pleosporales* sp. 1	*Dothideomycetes, Pleosporales*	ON063298	19
35	MAtR15	*Pleosporales* sp. 2	*Dothideomycetes, Pleosporales*	ON063299	2
36	MBwR12	*Pleosporales* sp. 3	*Dothideomycetes Pleosporales*	ON063300	17
37	MSuR12	*Westerdykella purpurea**	*Dothideomycetes, Pleosporales*	ON545805	20
38	MSuL5	*Xylaria* sp.	*Sordariomycetes, Xylariales*	ON063301	9
39	MBgL12	*Zopfiella* sp. 1	*Sordariomycetes, Sordariales*	ON063303	5
Total number of endophytic fungi isolated					**515**

* Indicates the new records of endophytic fungi from Sri Lanka

Molecular identification

The rDNA-ITS regions of representative isolates from thirty-nine (39) morphological groups were sequenced. The sequence data (ITS) of thirty-nine (39) isolates, including the thirteen (13) sterile forms, were compared with the ITS sequences in Genbank. Among them, thirty-one (31) taxa showed a sequence identity threshold of ≥99% with the ex-type and authenticated isolates in the NCBI database, and eight (8) taxa showed sequence identities below the threshold value. These eight taxa were not identified up to the species level, since they did not match any of the species in the database. However, the phylogenetic studies aided the identification and taxonomic placement of these taxa under specific orders or families. Thirteen morphological groups were considered sterile forms and identified as *Achroiostachys humicola*, *Dendryphiella* sp., *Magnaporthaceae* sp., *Magnaporthe salvinii*, *Nigrospora oryzae*, *Pseudothielavia terricola*, *Pyrenochaetopsis paucisetosa*, *Pleosporales* sp. 1, *Pleosporales* sp. 2 *Pleosporales* sp. 3, *Westerdykella purpurea*, *Xylaria* sp., and *Zopfiella* sp. 1. using the molecular data.

Phylogenetic analysis

In addition to BLAST analysis based on the NCBI database, MP phylogenetic trees were constructed to investigate the phylogenetic placement of the isolates used in this study with the ex-type and authenticated strains. These phylogenetic trees were used to confirm the species' identity when the BLAST results showed several homologous sequences with similar identities (results of individual generic trees are not shown). The phylogenetic tree was also used to confirm the taxonomic groups of the unidentified species from the BLAST homology. A total of thirty-nine (39) fungal isolates is summarized in Table 2. According to the USDA fungal database (Farr & Rossman 2022) and literature, 17species were new records in SL.

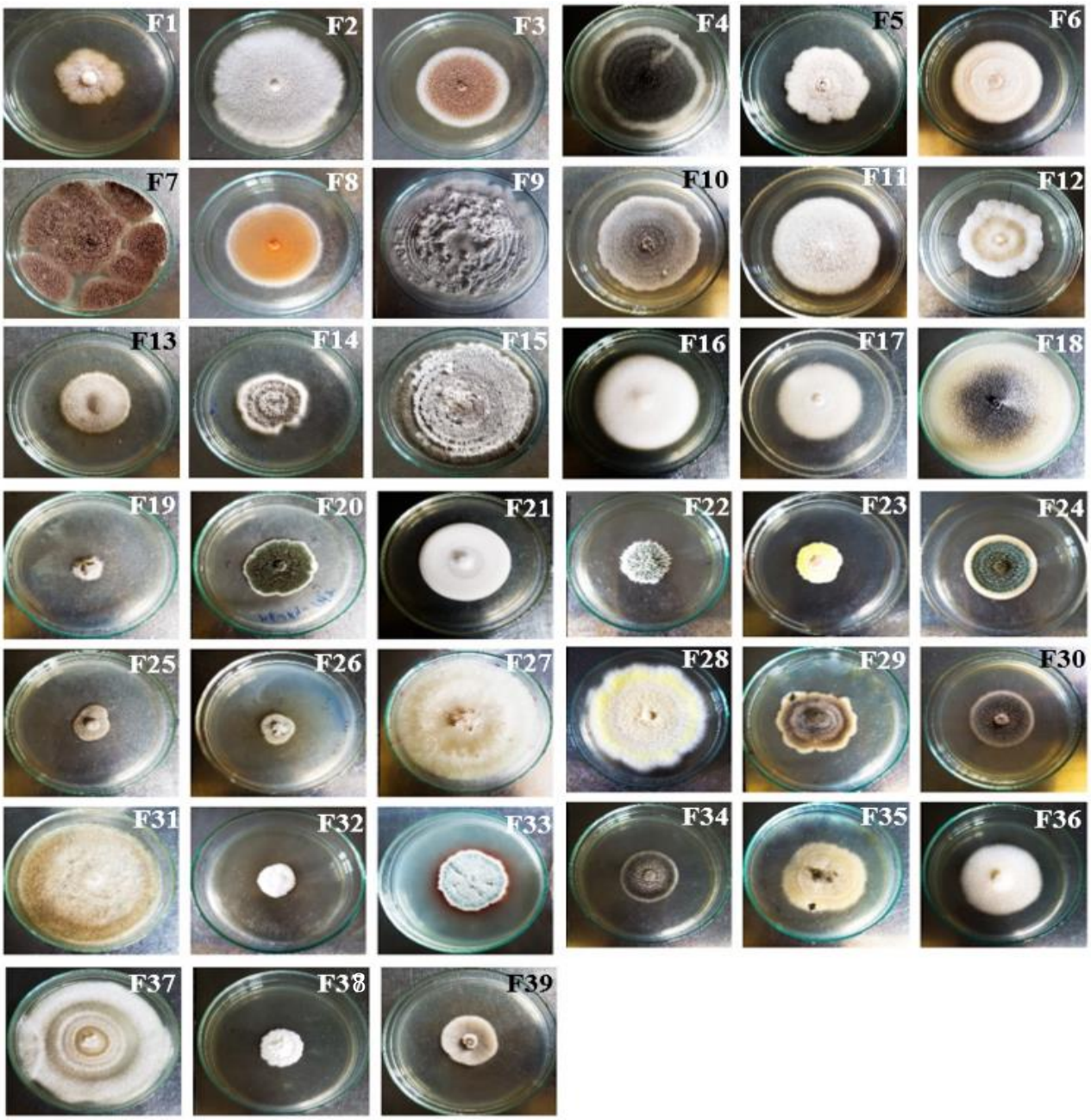


Fig. 3 – F1–F39 Colony morphology of endophytic fungal isolates recovered from Sri Lankan traditional and newly improved rice varieties. Seven days old fungal colonies in PDA medium. F1 *Achroiostachys humicola.* F2 *Aspergillus fischeri.* F3 *Aspergillus tereus*. F4 *Curvularia plantarum.* F5 *Daldinia eschscholtzii.* F6 *Dendryphiella* sp. F7 *Aspergillus assituensis*. F8 *Microdochium fisheri.* F9 *Bipolaris oryzae*. F10 *Exserohilum rostratum*. F11 *Fusarium annulatum.* F12 *Fusarium chlamydosporum.* F13 *Curvularia coicis.* F14 *Curvularia chiangmaiensis.* F15 *Curvularia lunata.* F16 *Fusarium falciforme.* F17 *Magnaporthaceae* sp. F18 *Magnaporthe salvinii.* F19 *Nigrospora oryzae.* F20 *Penicillium oxalicum.* F21 *Pseudothielavia terricola.* F22 *Talaromyces funiculosus.* F23 *Talaromyces liani.* F24 *Talaromyces purpureogenus.* F25 *Pyrenochaetopsis paucisetosa.* F26 *Ramichloridium apiculatum.* F27 *Rhizoctonia solani.* F28 *Talaromyces stipitatus.* F29 *Curvularia* sp 1. F30 *Pleosporales* sp. 1. F31 *Rhizopus microsporus.* F32 *Sarocladium oryzae*. F33 *Talaromyces veerkampii.* F34 *Pleosporales* sp. 2. F35 *Pleosporales* sp. 3. F36 *Westerdykella purpurea.* F37 *Zopfiella* sp. 1. F38 *Xylaria* sp. F39 *Zopfiella pilifera.*

The phylogenetic relationships between the EnF that were associated with four rice varieties collected from dry, wet, and intermediate zones of SL were studied by the ML-phylogram (Fig. 4).

Thirty-nine (39) sequences obtained in this study belonged to phyla Ascomycota, Basidiomycota and Zygomycota, and could be separated into nine monophyletic clades representing nine orders namely *Cantharellalles, Hypocreales, Eurotiales, Xylariales, Pleosporales, Mucorales, Sordariales, Mycosphaerellales,* and *Magnaporthales*. The highest number of species were in *Pleosporales* and *Eurotiales,* which included thirteen and nine species, respectively. The majority of the total number of isolates obtained in the whole study belonged to the genera of *Curvularia* (12.2%), *Microdochium* (9.9%), *Dendryphiella* (9.3%), and *Talaromyces* (8.9%) with the highest percentage of abundance (Fig. 5).

Even though most of the fungal species were isolated from all four rice varieties, some fungal species were associated with only one rice variety. For example, *Fusarium chlamydosporum* and *Ramichloridium apiculatum* were isolated only from the Bg 352 rice variety, while *Achroiostachys humicola* was exclusively found in the traditional rice variety Suwandel. *Aspergillus assituensis* was isolated only from At 362, and *Zopfiella pilifera* was isolated only from Bw 367. Most of the isolates, such as *Daldinia eschscholtzii, Magnaporthe salvinii*, *Aspergillus tereus, Talaromyces funiculosus, Exserohilum rostratum, Curvularia coicis,* and *Curvularia chiangmaiensis,* were isolated in both Suwandel and Bg352 only (Fig. 4). In addition, *Hypocreales* species were not recorded from the At 362 rice variety.

Phylogenetic placements of the EnF collected from dry, wet, and intermediate zones of SL were also indicated in the phylogenetic tree. Accordingly, no pattern was observed in the clades representing the climatic zones of rice cultivation. Instead, the EnF from almost all clades represented members from the dry, wet, and intermediate zones. However, certain species were uniquely isolated from the rice varieties in particular cultivation zones. For example, *Aspergillus assituensis, Talaromyces liani, Pleosporales* sp. 2, *Ramichloridium apiculatum, Zopfiella pilifera,* and *Zopfiella* sp. 1 were exclusively isolated from the wet zone plants, while *Fusarium chlamydosporum* and *Magnaporthe salvinii* were isolated from both the wet and intermediate zone, but not in the dry zone. In the intermediate zone, *Talaromyces funiculosus, Talaromyces stipitatus* and *Pleosporales* sp. 3 were absent. Similarly, *Achroiostachys humicola* and *Aspergillus tereus* were absent in the wet zone, indicating that there is no clear divergence in the clades of EnF assemblages in rice varieties that affected their distribution in different geo-climatic zones of rice cultivation.

Endophytic fungal colonization, diversity, and their dominance in four common rice varieties of Sri Lanka

The extent of colonization by EnF varied among the different rice varieties from different geographical locations. The CPs were higher in Suwandel and Bg 352, whereas CPs were comparatively low in At 362 and Bw 367. The fungal CPs differed among the four rice varieties collected from four geographical locations and the two cultivation seasons. The fungal taxa, such as *Aspergillus tereus*, *Microdochium fisheri*, *Dendryphiella* sp., *Curvularia lunata, Fusarium falciforme, Penicillium oxalicum*, *Rhizopus microsporus, Sarocladium oryzae*, and *Talaromyces purpureogenus* were commonly found in all four rice varieties with a higher percentage of dominance. But fungal taxa, such as *Aspergillus assituensis*, *Zopfiella* sp. 1, and *Ramichloridium apiculatum,* were found less frequently and isolated only once. However, all the rice varieties were found to be associated with various EnF with varying dominance. Shannon-Weiner's and Simpson's diversity index values indicated that EnF associated with four commonly cultivated rice varieties in SL showed great diversity, varying with rice variety, geographical location, and cultivation seasons (Fig. 6).

Colonization, diversity, and dominance of endophytic fungi of rice varieties in dry, intermediate, and wet zones in Sri Lanka

Anuradhapura (Dry zone)

A total of thirty-five (35) isolates were acquired from Anuradhapura from all four rice varieties during the Maha season, while seventy-seven (77) isolates were obtained during the Yala season. In

the dry zone plants, the CPs of EnF were high in Suwandel (25%) during the Maha season, whereas during the Yala season the highest CPs were observed in Bg 352 (37%). Total colonization percentages in all rice varieties were comparatively higher during the Yala than during the Maha season in the dry zone plants (Fig. 6).

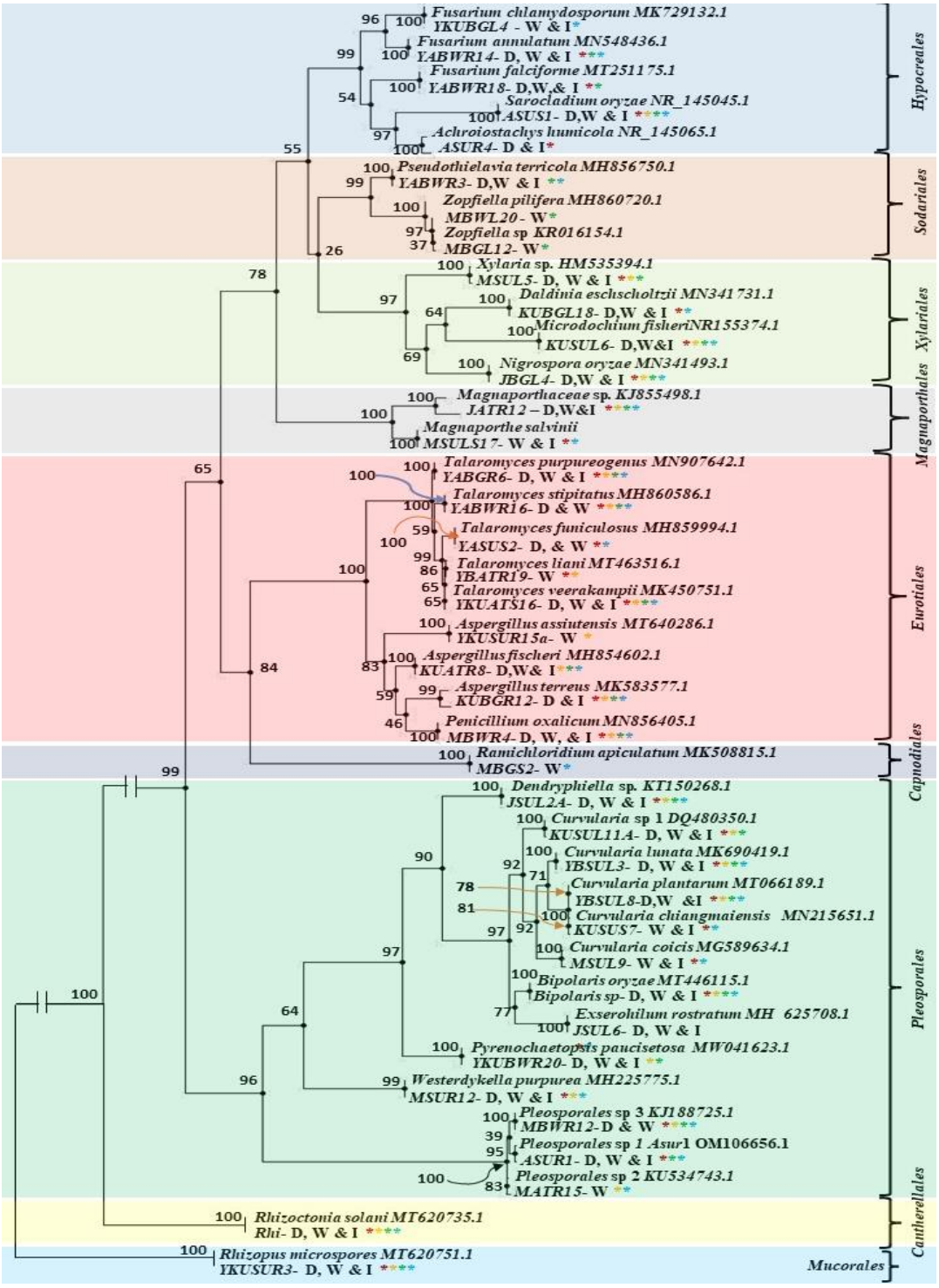


Fig. 4 – Maximum likelihood (-10620.686803) phylogram showing the placement of all the isolates based on the sequences of the ITS region of rDNA with reference isolates. The tree was constructed using the RAXML-HPC Black Box model for pairwise distance measurement and rooted with *Rhizopus microsporus* (Accession No: ON063291). Only bootstrap values of >50% (1,000 replicates) are shown above the branches. D-dry zone, W-wet zone, I- intermediate zone; ***** - Suwandel, * - At 362, *- Bw 367, *- Bg 352.

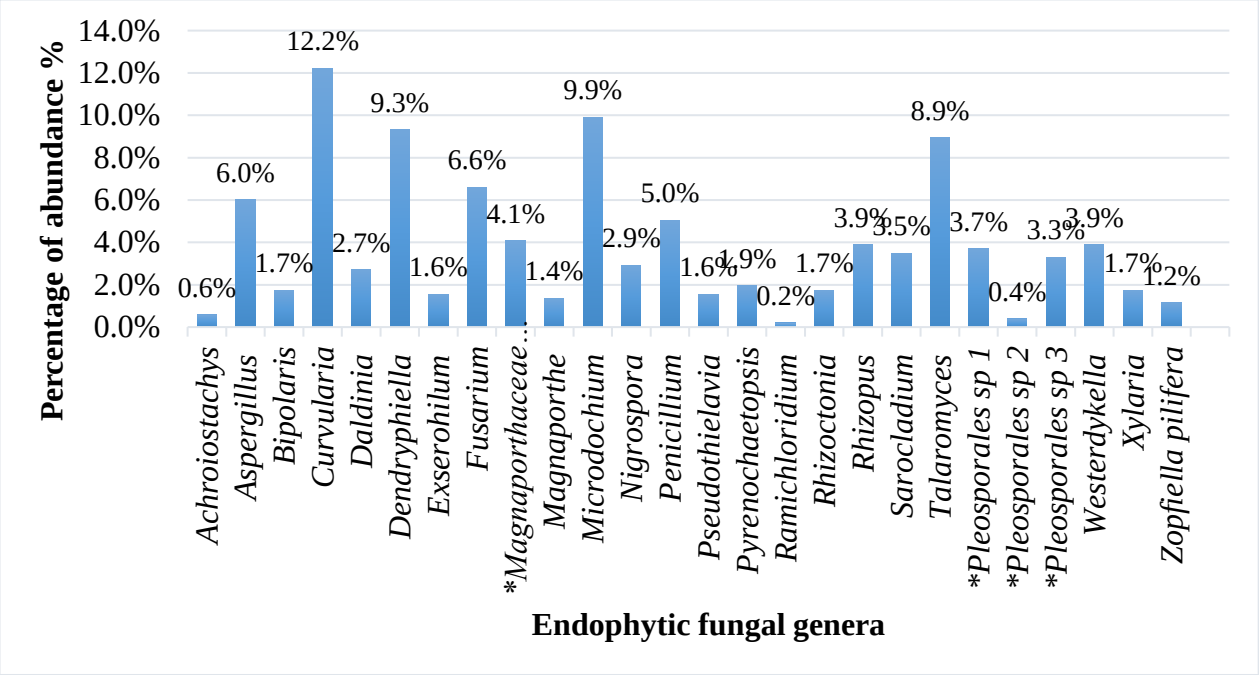


Fig. 5 – Endophytic fungal taxa in four rice varieties collected from four different geographical locations in Sri Lanka. Data is based on the total number of genera and the total number of isolates (515) obtained from the whole study; *denotes undescribed fungal genera; genus names of identified fungi are indicated below the corresponding column assigned.

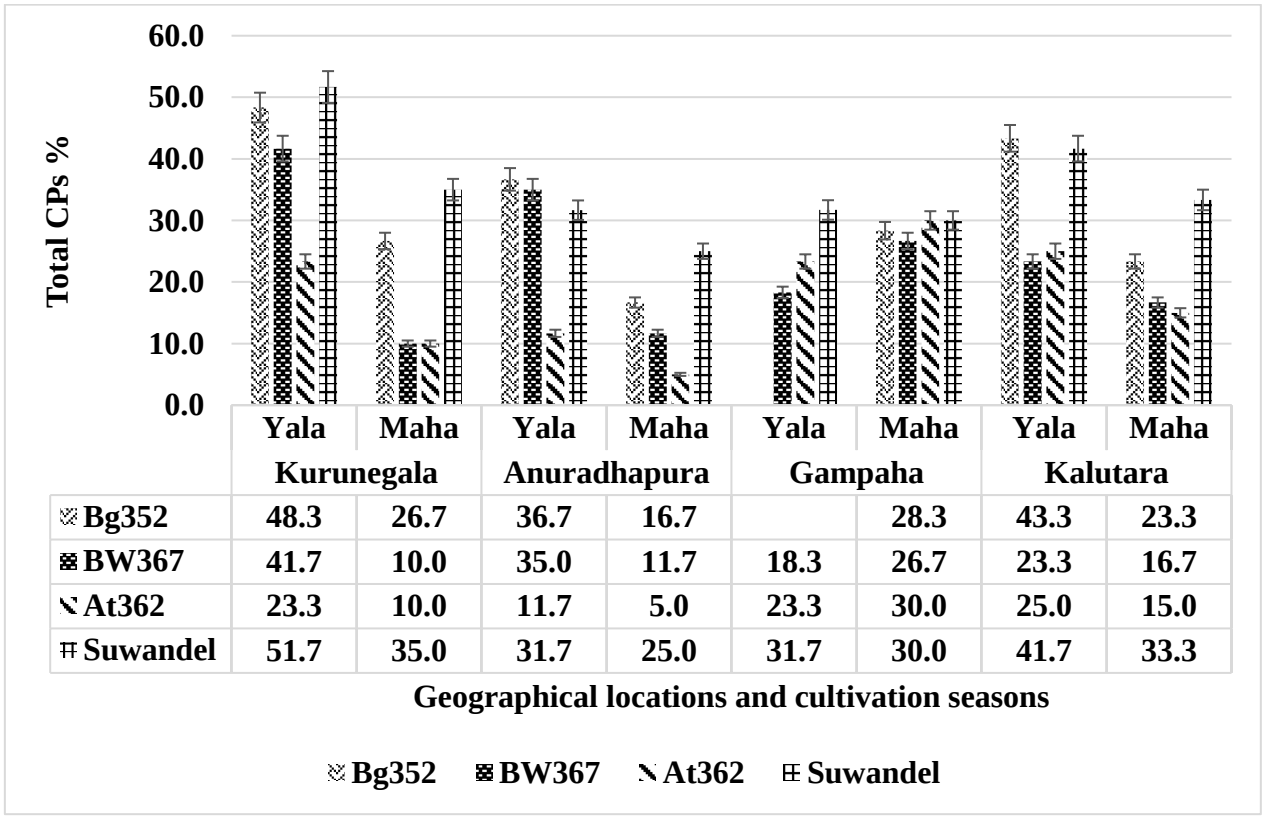


Fig. 6 – Total Percentage of colonization by endophytic fungi in plant tissues of rice varieties collected from different geographical locations. Data is based on a total number of sixty (60) tissue segments from a single rice variety incubated by placing 20 segments of each leaf, stem, and root sample in each trial. Bars represent the standard error percentage.

Suwandel collected during the Maha season yielded the maximum number of taxa (12), while both Suwandel and Bw 367 inhabited the highest number of different taxa (10) during the Yala season. The lowest number of taxa were isolated from At 362 during both Yala (7) and Maha (2) seasons out of the other rice varieties.

The results showed that fungal endophytes, such as *Microdochium fisheri* (8%), *Pleosporales* sp. 1 (7%), *Rhizoctonia solani* (7%), *Rhizopus microspores* (7%), *Talaromyces veerkampii* (7%), *Fusarium falciforme* (7%), *Penicillium oxalicum* (7%), and *Aspergillus tereus* (5%) were the most dominant fungi from the dry zone plants during both seasons, with a comparatively higher percentage of dominance.

In Anuradhapura, Simpson's (D = 0.9074) and Shannon-Weiner's (H' = 2.428) diversity indices were higher in Suwandel during the Maha season, and during the Yala season, the results showed that the highest Simpson's (D = 0.8771) and Shannon-Weiner's (H' = 2.231) indices were displayed in Bg 352, which showed higher species diversity than the traditional rice variety. The lowest species diversity was observed in At 362 collected during the Maha season with Simpson's (D = 0.4444) and Shannon's (H' = 0.6365) indices. However, during the Yala season, species diversity of rice varieties At 362, Bw 367, and Suwandel were nearly the same, with similar Simpson's and Shannon-Weiner's indices (Figs 7, 8).

Kurunegala (Intermediate zone)

A total of forty-six (46), and one hundred and six (106) EnF isolates were obtained during the Maha season and Yala season respectively from the plant parts of all four rice varieties collected from the intermediate zone. The CPs of EnF were high in Suwandel (35%), during the Maha season. Similarly, during the Yala season, also the results showed the highest CPs in Suwandel (52%) (Fig. 6). The highest number of taxa was obtained from Suwandel during both the Maha (11) and Yala (18) seasons, respectively.

The most dominant fungal endophytes in the intermediate zone rice plants were *Aspergillus tereus* (5%), *Aspergillus fischeri* (5%), *Curvularia coicis* (7%), *Curvularia lunata* (7%), *Daldinia eschscholtzii* (5%), *Dendryphiella* sp. (7%), *Magnaporthe salvinii* (7%), *Rhizopus microsporus* (7%), *Nigrospora oryzae* (9%), *Rhizopus microspores* (7%), *Talaromyces purpureogenus* (7%), and *Westerdykella purpurea* (5%).

In Kurunegala, the highest Simpson's (D = 0.8926 and 0.922) and Shannon–Wiener's (H' = 2.303 and 2.718) indices were observed in Suwandel during both the Maha and Yala seasons respectively, while the lowest Simpsons (D = 0 and 0.875) and Shannon–Wiener's (H' = 0 and 2.186) indices were observed in Bw 367, collected during the Maha and Yala seasons respectively (Figs 7, 8).

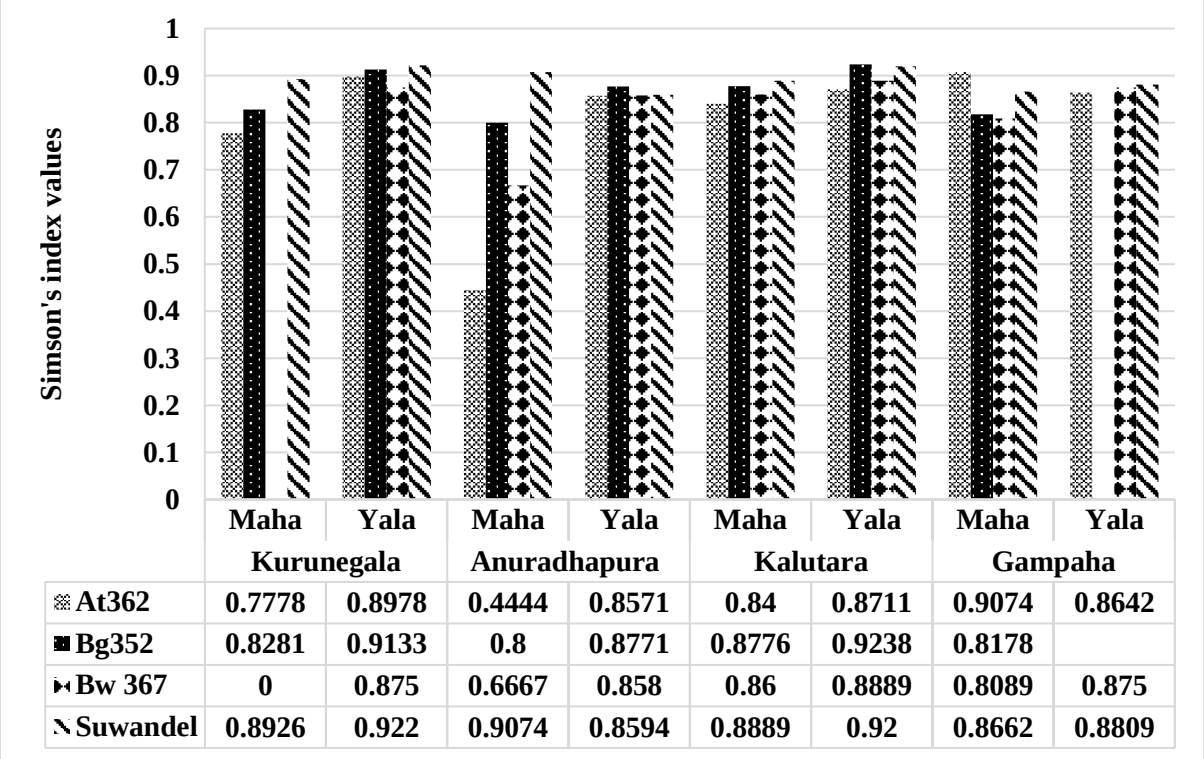
Gampaha and Kalutara (wet zone)

Samples of rice varieties were collected from two locations in the wet zone due to the unavailability of the Bg 352 rice variety in Gampaha during the Yala season. The number of isolates obtained from Gampaha, and Kalutara during the Maha season was sixty-nine (69) and fifty-six (56) respectively. During the Maha and Yala seasons, Suwandel collected from both Gampaha and Kalutara (Fig. 6) showed the highest CPs. When comparing the two sites in the wet zone, the number of taxa obtained from Suwandel collected from both Gampaha and Kalutara showed a maximum number of taxa of 12 and 10 respectively, higher than other rice varieties. However, during Yala season the highest number of taxa (22) was obtained from Bg 352.

Fungal endophytes such as *Daldinia eschscholtzii* (10%), *Microdochium fisheri* (10%), *Bipolaris oryzae* (7%), *Curvularia lunata* (7%), *Dendryphiella* sp. (7%), *Exserohilum rostratum* (5%), *Sarocladium oryzae* (5%), *Pyrenochaetopsis paucisetosa* (5%), and *Magnaporthaceae* sp. (5%) were the most dominant species in the wet zone-rice plants from both sites.

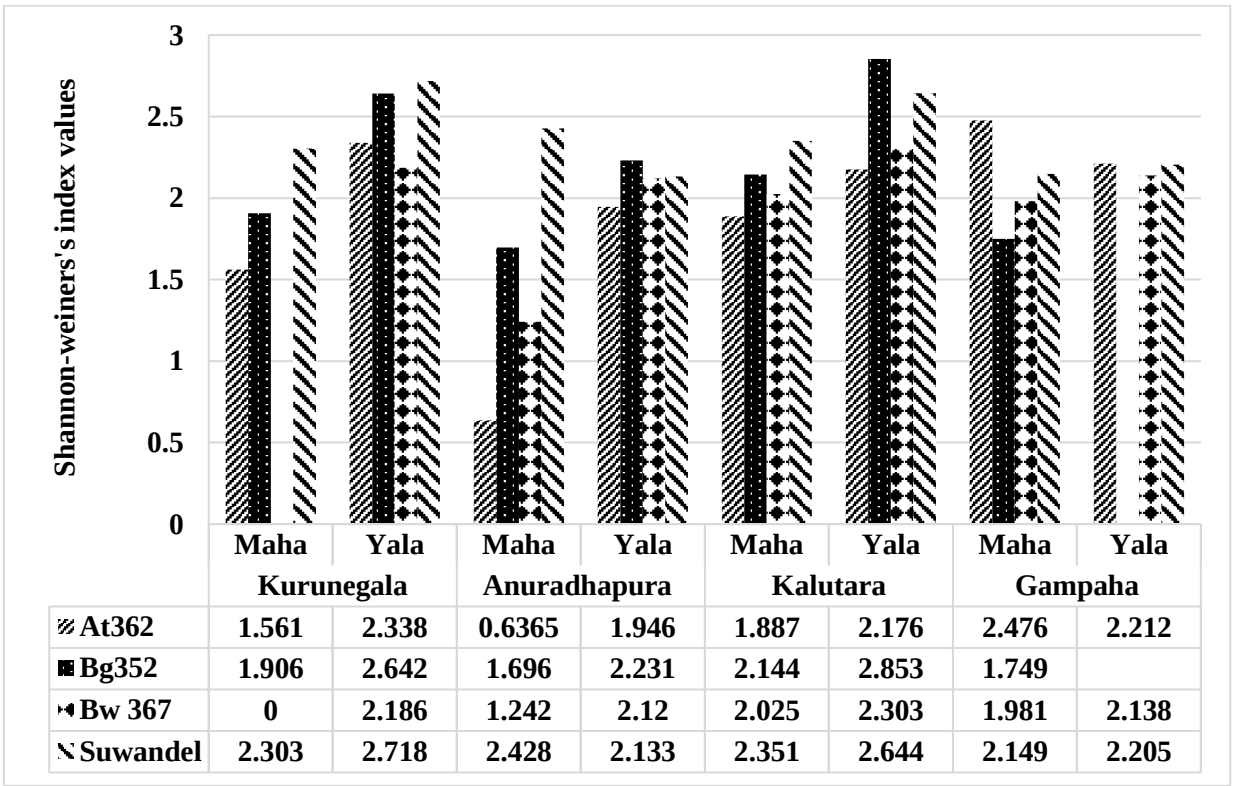
The Simpson's indices of EnF were greatest in Suwandel (D = 0.8809) from Gampaha, while At 362 showed the highest Shannon-Weiner's diversity (H' = 2.212). Bg 352 showed maximum index values of Simpson's (D = 0.9238) and Shannon–Wiener's diversity (H' = 2.853) in Kalutara

during the Yala season, and diversity indices were greater in Suwandel collected from Gampaha during the Maha season (D = 0.8889, H' = 2.351) (Figs 7, 8).



	Kurunegala		Anuradhapura		Kalutara		Gampaha	
	Maha	Yala	Maha	Yala	Maha	Yala	Maha	Yala
At362	0.7778	0.8978	0.4444	0.8571	0.84	0.8711	0.9074	0.8642
Bg352	0.8281	0.9133	0.8	0.8771	0.8776	0.9238	0.8178	
Bw 367	0	0.875	0.6667	0.858	0.86	0.8889	0.8089	0.875
Suwandel	0.8926	0.922	0.9074	0.8594	0.8889	0.92	0.8662	0.8809

Fig. 7 – Simpson's indices of endophytic fungal assemblages of four rice varieties collected from four locations during the Maha and Yala season (2019).



	Kurunegala		Anuradhapura		Kalutara		Gampaha	
	Maha	Yala	Maha	Yala	Maha	Yala	Maha	Yala
At362	1.561	2.338	0.6365	1.946	1.887	2.176	2.476	2.212
Bg352	1.906	2.642	1.696	2.231	2.144	2.853	1.749	
Bw 367	0	2.186	1.242	2.12	2.025	2.303	1.981	2.138
Suwandel	2.303	2.718	2.428	2.133	2.351	2.644	2.149	2.205

Fig. 8 – Shannon-Weiner's indices of endophytic fungal assemblages of four rice varieties collected from four locations during the Maha and Yala season (2019).

Discussion

Studies on colonization, diversity, dominance, and phylogenetic relationships of EnF isolated from four varieties of rice, i.e., Suwandel, At 362, Bw 367, and Bg 352, that are commonly cultivated in SL were conducted as a comprehensive study. Understanding such facets of EnF is essential for their efficient exploitation as alternatives to agrochemicals.

Amongst the endophytic isolates, thirty-nine morphologically different EnF groups were identified based on their colony and micro-morphological characteristics. Some morphological groups were sterile forms, and their identification is reported as a widespread problem (Guo et al. 2000). Sequencing of fungal barcode ITS region (Schoch et al. 2012) has been proposed as a solution for the identification of sterile EnF in previous work (Doss & Welty 1995, Lacap et al. 2003, Brader et al. 2017, Deyett & Rolshausen 2020). Results demonstrate that the use of ITS sequence data is an effective method for classifying most of the fungal isolates up to the generic level. However, the use of ITS sequence data is inadequate to identify all isolates to the species level. Therefore, sequences of additional barcoding genes should be utilized in phylogenetic analysis. The inconsiderate use of species names from the GenBank database without confirming their identifications has led to the misidentification of many EnF species (Ko Ko et al. 2011). Hence, sequences of EnF obtained from the present study were compared with ex-type and authenticated sequences. Maximum parsimony and maximum likelihood generic trees were constructed using ex-types and authenticated sequences for the different genera obtained in the present study (Results not shown). This has also been reported in previous studies, where the phylogenetic data along with the morphological characteristics is a more effective method in EnF identification. (Rehner & Uecker 1994, Rivera-Orduña et al. 2011, Manamgoda et al. 2012, Manganyi et al. 2018, Radiastuti et al. 2019, Dos Santos Oliveira et al. 2020, Barberis et al. 2021, Luo et al. 2022).

The literature available on phylogenetic relationships of EnF assemblages in different rice varieties from different cultivation zones of SL is limited. Therefore, analyses carried out in the present study provide insight into the phylogenetic relationships of the EnF communities associated with the rice varieties and the cultivation zones (dry, intermediate, and wet zones) in SL. Endophytic fungal isolates from dry, wet, and intermediate zones were closely related, with a few exceptions, where some species were isolated only from some cultivation zones. A few species were also exclusively isolated from some rice varieties, supporting the concept that host varieties and cultivation sites may play a role in determining the evolutionary relationships and species composition of EnF, as reported in previous studies (Márquez et al. 2012, Pili et al. 2016).

Most of the fungi in the present rice endophyte collection belong to the phylum Ascomycota, while some belonged to the phyla Basidiomycota and Zygomycota. Amongst the Ascomycetes, most species belonged to the orders *Pleosporales* and *Eurotiales*, representing larger clades in the tree. Pili et al. (2016) also showed that most species isolated from irrigated rice varieties Basmati 370 (Kenya Pishori), IR279380-1 in Kenya, belonged to orders *Pleosporales* and *Eurotiales*, which is similar to the results of the present study. The phylogenetic tree also showed the placement of the unidentified species. Also, some non-sporulated isolates such as ASUR1, MATR15, and MBWR12 were identified as *Pleosporales* sp. 1, 2, and 3, and JATR12 was identified as a species belonging to the family *Magnaporthaceae* according to phylogeny. Their identification was further confirmed by the phylogenetic tree, as they aligned with other closely related species in their respective clades. Seventeen (17) EnF species were identified as novel records in SL, which is one of the remarkable findings that would support the fungal taxonomy of rice-EnF in Sri Lanka.

Studies on EnF associated with cultivated rice in China, India, and Italy have reported the patterns of colonization, diversity, distribution, and population of EnF from different plant parts of rice varieties collected from different geographical locations and cultivation seasons (Tian et al. 2004, Naik et al. 2009, Vallino et al. 2009, Chadha et al. 2015, Lugtenberg et al. 2016). Colonization, dominance, and species composition of EnF in different plant tissues, such as leaves, stems, and roots of Sri Lankan traditional rice varieties Suwandel, Kaluheenati, Kuruluthuda, and Herath Banda, have also been reported (Ponnawila & Deshappriya 2014, Atugala & Deshappriya 2015, Wijesooriya & Deshappriya 2016). However, the present study provides information on the EnF assemblages

associated with more widely cultivated, newly improved rice varieties. A comparison of fungal species in traditional and newly improved rice varieties has not been conducted before. The present study provides additional information on these aspects of EnF in Sri Lankan rice varieties.

According to the results, there were no specific patterns in the extent of total colonization percentages of EnF in the selected rice varieties sampled from different geographical locations or climatic zones. However, the colonization rates of EnF were greater in the traditional rice variety Suwandel collected from most locations. In addition, the overall colonization of all rice varieties was greater during the Yala season, which had lower rainfall and a higher average temperature than that in the Maha season, which was consistent with the previous studies that found seasonal changes affect the colonization of plant species by endophytic mycobiota (Wilson & Carroll 1994, Lodge & Cantrell 1995, Suryanarayanan et al. 1998).

In this study, thirty-nine fungal species were isolated, and *Aspergillus tereus*, *Microdochium fisheri*, *Dendryphiella* sp., *Curvularia lunata*, *Fusarium falciforme*, *Penicillium oxalicum*, *Rhizopus microsporus*, *Sarocladium oryzae*, and *Talaromyces purpureogenus* were the most frequently isolated species from all four rice varieties collected from selected locations. The fact that many species can be isolated from a given host, but only a very few species are present in a considerable number, has been highlighted by Petrini et al. (1992), which conforms to the findings of this study. Petrini et al. (1992) showed that out of the thirty-one species isolated from rice varieties, only a few species were major colonizers. This pattern of colonization by two or three dominant taxa by a host due to minor opportunistic infections is also typical of EnF colonization in many perennials and vascular plants (Petrini et al. 1992). It is also an indication of potential co-evolution between the dominant EnF and their hosts, as suggested by Carroll (1988). The present study also showed that even in newly improved rice varieties, EnF follow a pattern of colonization remarkably similar to the traditional rice varieties.

Most of the isolated fungal taxa in this study were reported as endophytes of rice in previous studies, while some of them were common genera of soil fungi, e.g., *Fusarium*, *Penicillium*, and *Rhizoctonia* sp. These fungi are soil-inhabitant free-living saprophytes that can also be opportunistic root symbionts or pathogens in rice varieties (Fisher et al. 1992, Tian et al. 2004). Previous studies in SL have reported several species of *Aspergillus*, *Curvularia*, *Fusarium*, *Penicillium*, *Rhizoctonia*, and *Rhizopus* to be endophytes of the traditional rice variety Suwandel (Ponnawila & Deshappriya 2014, Atugala & Deshappriya 2015, Wijesooriya & Deshappriya 2016). These EnF species were also detected in the variety Suwandel used in the present study. Moreover, *Aspergillus tereus*, *Talaromyces purpureogenus*, and *Westerdykella purpurea* were reported in rice varieties Basmati 370 (Kenya Pishori), IR279380-1 (Pili et al. 2016), and were also detected in the Sri Lankan rice varieties used in the present study. These species have been detected to be associated with medicinal plant species, such as sunflower (Waqas et al. 2015) and *Tylophora ovata* (Padmathilake et al. 2017), which showed the diverse ability of EnF to colonize several host species or varieties. In addition, a few rice-EnF, such as *Bipolaris oryzae* (Liu et al. 2021), *Sarocladium oryzae* (Lanoiselet et al. 2012), *Rhizoctonia solani* (Lee & Rush 1983), and *Rhizopus microsporus* (Lennartsson et al. 2014), isolated in the present study, were also identified as opportunistic rice pathogens in previous studies.

Interestingly, a small proportion of singletons (i.e., endophytes isolated only once), i.e., *Aspergillus assituensis*, *Ramichloridium apiculatum*, and *Zopfiella* sp. 1 were detected in this study. Several researchers have reported that singletons represent a high proportion of endophytes recovered from different plant species (Gallery et al. 2007, Joshee et al. 2009). Yuan et al. (2010) showed that a considerable proportion of singletons were obtained from the stems of a wild rice variety in China. Even though they are recognized as opportunistic colonizers or contaminants and thus excluded from estimates of total species richness, Clay (2004) and Stone et al. (2004) stated that they should not be ignored when inferring the ecological significance of an EnF community due to their potential functional and biotechnological applications. Indeed, singletons could also represent novel species unique to the host plant, necessitating their presence in the estimates of total endophytic biodiversity. In the present study, singletons such as *Aspergillus assituensis* and *Zopfiella pilifera* were also identified as novel records of SL.

Diversity analysis of fungal endophytes in rice varieties by Shannon-Weiner and Simpson's indices showed that the diversity indices varied between rice varieties from different cultivation zones and cultivation seasons. Nevertheless, any specific patterns in the diversity changes were not observed in the present study. According to the literature, both Shannon-Weiner's and Simpson's diversity indices rose when the species richness and evenness of a community increased (Uzma et al. 2016). Several studies have also shown that the abundance, diversity, dominance, and species composition of endophytic assemblages (Ribeiro et al. 2018, Rashmi et al. 2019, Chen et al. 2020) vary according to host species (Joshee et al. 2009), age, type of tissue (Unterseher et al. 2007, Guo et al. 2008), site characteristics (Gao et al. 2005, Arnold et al. 2007, Helander et al. 2007), local microclimate conditions (Helander et al. 2007, Guo et al. 2008, Hoffman & Arnold 2008), and anthropogenic factors (Helander et al. 1994, Ranta et al. 1995). The above-mentioned reasons might contribute to the results obtained in the present study, as no definite pattern was observed in the diversity indices of EnF in the rice varieties obtained from the different geo-climatic zones and seasons in SL.

In summary, culturing and molecular characterization indicated that Sri Lankan traditional and newly improved rice varieties constitute fungal consortia, including several ancestries of EnF. It is evident that host plant characteristics, seasonal climatic conditions, and geographical locations contribute to the colonization, dominance, and diversity of EnF associated with the rice varieties selected for this study. ITS sequencing and phylogenetic studies provided an accurate insight into the diversity of the EnF assemblages in the rice ecosystems of SL and aided in understanding the evolutionary relationships between EnF in commonly cultivated rice varieties collected from different geo-climatic zones of SL. Interestingly, out of the thirty-nine (39) fungal taxa that were found to be associated with rice, seventeen (17) are novel records of SL. However, it should be noted that ITS sequence data undergoes taxonomic changes, and fungal identification based on ITS sequence data can easily be revised and updated, as taxonomic changes are frequently made (Buehler et al. 2017). This study will contribute to the understanding of the ecology, evolution, and taxonomy of EnF assemblages in selected newly improved rice varieties of Sri Lanka.

Acknowledgments

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Supplementary data

Supplementary data associated with this article - Percentage Dominance of endophytic fungal isolates from rice varieties collected from selected locations during the Yala and Maha seasons.

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