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OPINION

# Methods used for the study of endophytic fungi: a review on methodologies and challenges, and associated tips

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## Abstract

Endophytic fungi are microorganisms that colonize the interior of plant tissues (e.g. leaves, seeds, stem, trunk, roots, fruits, flowers) in intracellular and/or extracellular spaces without causing symptoms of disease in host plants. These microorganisms have been isolated from plant species in a wide variety of habitats worldwide, and it is estimated that all terrestrial plants are colonized by one or more species of endophytic fungus. In addition, these microorganisms have been drawing the attention of researchers because of their ability to synthesize a wide range of bioactive molecules with potential for applications in agriculture, medicine and biotechnology. However, several obstacles come up when studying the diversity and chemical potential of endophytic fungi. For example, the usage of an inappropriate surface disinfection method for plant tissue may not eliminate the epiphytic microbiota or may end up interfering with the endophytic mycobiota, which consequently generates erroneous results. Moreover, the composition of the culture medium and the culture conditions can favor the growth of certain species and inhibit others, which generates underestimated results. Other inconsistencies can arise from the fungus misidentification and consequent exploration of its chemical potential. Based on the methodological biases that may occur at all stages of studies dealing with endophytic fungi, the objective of this review is to discuss the main methods employed in these studies as well as highlight the challenges derived from the different approaches. We also report associated tips to help future studies on endophytic fungi as a contribution.

**Keywords** Endophyte · ITS · Molecular identification · Diversity · Mycobiome · Symbiosis

## Introduction

Fungi are eukaryotic, heterotrophic organisms that grow as elongated, polarized cells (hyphae) or budding cells called yeasts, which reproduction occurs through meiotic and/or mitotic processes (Naranjo-Ortiz et al. 2019). The lifestyle of these microorganisms evolved independently in the Tree of Life (Hawksworth and Lucking 2018). Thus, this realm comprises one of the greatest species biodiversity among eukaryotic organisms (Willis 2018). Due to the complexity of their morphological structures and lifestyle that often depends on other organisms, the knowledge on distribution and diversity of fungi, particularly endophytic fungi, remains mostly unknown.

Endophytic fungi or fungal endophytes are a complex group of polyphyletic organisms (Chi et al. 2019). The constituent taxa are able to colonize intra/extracellularly the plant tissues (e.g. stem, flowers, leaves, fruits, roots) (Li et al. 2016a, b) during part or their entire life cycle without causing an apparent manifestation of disease in the tissue colonized (Bacon and White 2000).

In general, the methods employed to study endophytes are relatively simple, but involve complex steps that must be strictly followed to avoid misinterpretation (Fig. 1). After the plant material collection, the first step is the superficial disinfection of the sampled tissue to eliminate the epiphytic microbiota (Li et al. 2016a, b; Yao et al. 2017), followed by culture-dependent (Szűcs et al. 2018; Yao et al. 2019; Du et al. 2020; Fan et al. 2020) and/or independent techniques (Yao et al. 2019).

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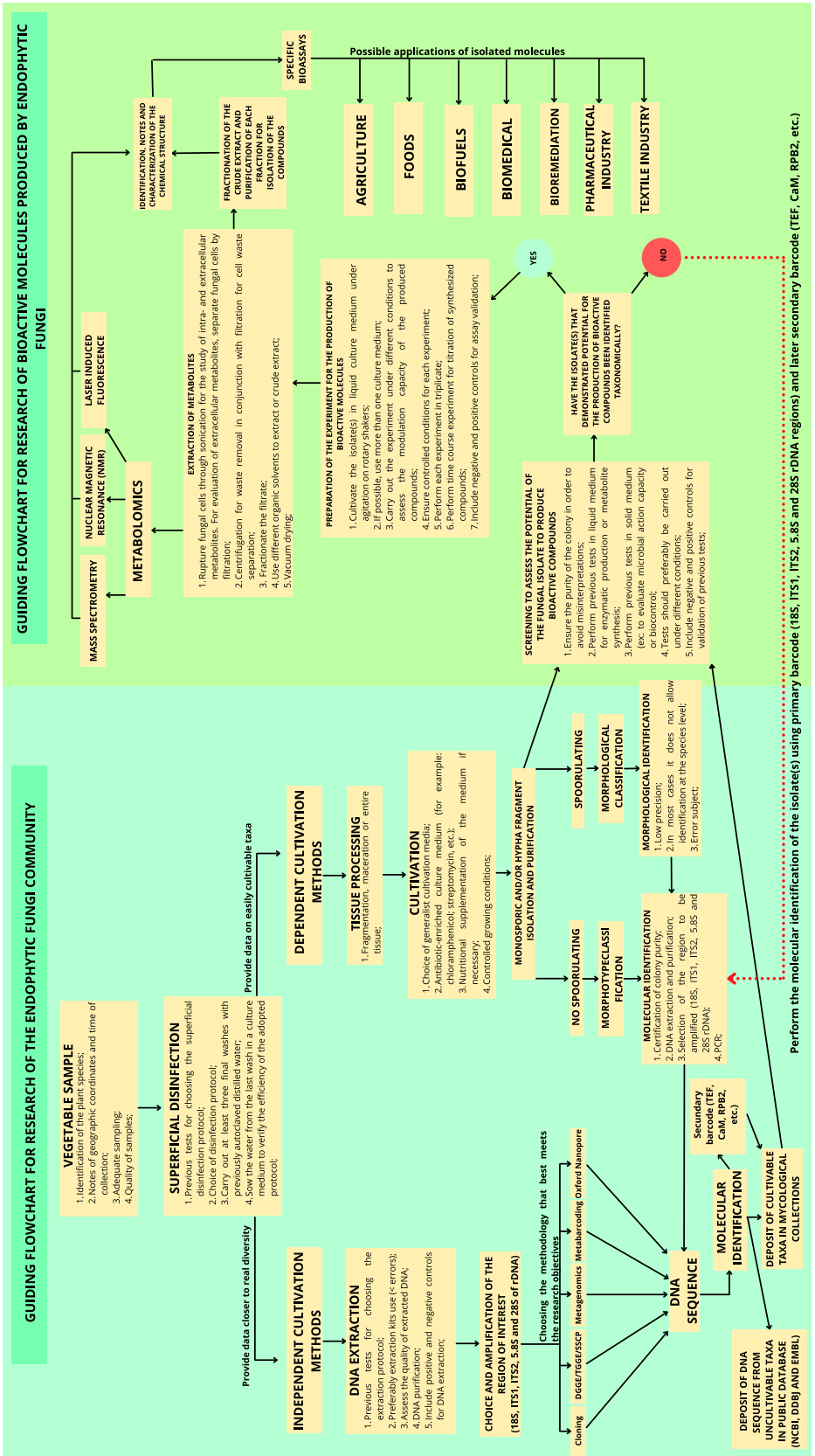


**Fig. 1** Flowchart for studying the diversity of endophytic fungi and their potential for producing bioactive compounds

Over the decades, the isolation of endophytic fungi was based on culture-dependent methodologies (Szűcs et al. 2018; Hamzah et al. 2018; Gong et al. 2019). For this, the plant samples are fragmented and distributed onto culture medium, incubated under controlled conditions and, after the fungal growth, the colonies are purified and the isolate identified using morphological characteristics or molecular analyses (Ibrahim et al. 2017; Pietro-Souza et al. 2017; Yao et al. 2017).

It is well known that culture-based methods do not reflect the real diversity of fungi in a niche (Fan et al. 2020; Lücking et al. 2020), since some of these microorganisms cannot be cultivated under laboratory conditions (Fan et al. 2020; Wu et al. 2019). In addition, identification of isolates using morphological features can provide misidentification, since fungi of different species have similar type of structure (Lücking et al. 2020), such as hyphae consisted of one or more cells surrounded by a tubular cell wall. Furthermore, some of these methods require modifications, including enriched-culture medium with a certain substrate to increase the fungal species diversity (Mourad et al. 2018), coupled with the use of molecular methods for identification of isolates (Noriler et al. 2018).

Despite advances in methodologies to study endophytic fungi, several barriers come up at all stages of commonly used protocols, requiring well-established strategies to avoid misinterpretation. Here, we discuss some methods mostly used to study endophytic fungi, emphasizing the main limitations derived from the different approaches, with alternatives to circumvent and/or minimize the existing barriers.

## Collection and sampling of plant material

The first step to study endophytic microorganisms is the collection of plant material. However, there is no universal consensus on the adequate number of individuals and/or samples per individual that should be considered. It is strongly recommended a representative sampling according to the objectives proposed by the study. Considering the geographic factor, Yao et al. (2019) accounted eight individuals per plant species sampled and recommended a minimum distance of at least 50 m between each plant species in studies on diversity of endophytic fungi of different tropical mangroves. Wang et al. (2019) aimed to describe the three-years-old mycobiota of *Zanthoxylum bungeanum* and for these ten specimens were sampled excluding the spatial criteria. Fan et al. (2020) studied the endophytic mycobiome of the cultivated *Huperzia serrata* and three specimens were randomly chosen. Therefore, the number of plant individuals sampled depends on the objective of the research, but the quantity and quality of sampling plus biotic and abiotic factors that can interfere with the endophytic mycobiota must be considered (Fig. 2).

Interestingly, most fungal genera and/or species are found in specific host plant species probably because the phytochemistry and micro/macronutrient contents present in the leaf tissue that acts by selecting the fungal species for colonization (Arnold et al. 2003).

Regarding sampling by the individual, a number of studies (Li et al. 2016a, b; Yao et al. 2017, 2019; Szűcs et al. 2018; Du et al. 2020; Fan et al. 2020) suggest > 5 samples per individual, but extraordinary cases should be analyzed. Biotic and abiotic factors that can cause interference on the endophytic fungi community harbored by the sampled individual should be accounted. In some cases, it is necessary to have a composite and representative sample (Fig. 3).

Environmental conditions are responsible for species distribution not only in a macroscale, but also in a microscale, and even within a local site as shown in Fig. 3A. These variations within microenvironments expose natural population such as fungi to mosaics of resource availability and different abiotic (e.g. temperature, humidity, light intensity, availability of nutrients) and biotic conditions (e.g. interaction with nodes of the same species and other species) (Kluting et al. 2019; Denney et al. 2020; Hurtado-McCormick et al. 2019). Therefore, these factors must be taken into account during sampling in endophytic fungi studies.

After collecting the plant material, the samples should be placed in a sterile plastic container or bag, transferred to a cool box at a controlled temperature, and preferably processed within 24 h after sampling (Lundberg et al. 2012; Sharma et al. 2016; Li et al. 2016a, b; Ibrahim et al. 2017, 2021; Pietro-Souza et al. 2017; Yao et al. 2017, 2019; Szűcs et al. 2018; Hamzah et al. 2018; Gong et al. 2019; Dhayanithy et al. 2019; Thi et al. 2019; Jayatilake and Munasinghe 2020; Du et al. 2020; Fan et al. 2020; Chowdappa et al. 2020) under aseptic conditions.

## Surface disinfection of plant material

Since the discovery of taxol in an endophytic fungus associated with *Taxus* spp. (Stierle et al. 1993), endophytic fungi have been drawing the attention of the scientific community either by the species diversity and/or the capacity to synthesize bioactive molecules. Nevertheless, several methodological obstacles appear when studying endophytes, including the complete removal of microorganisms that make up the epiphytic microbiota. Effective methods of surface disinfection to remove the epiphytic microbiota are mandatory first steps for studying endophytic microorganisms, which includes chemical or physical procedures.

The chemical sterilization of the sample surface is the most used method for removing microorganisms from the

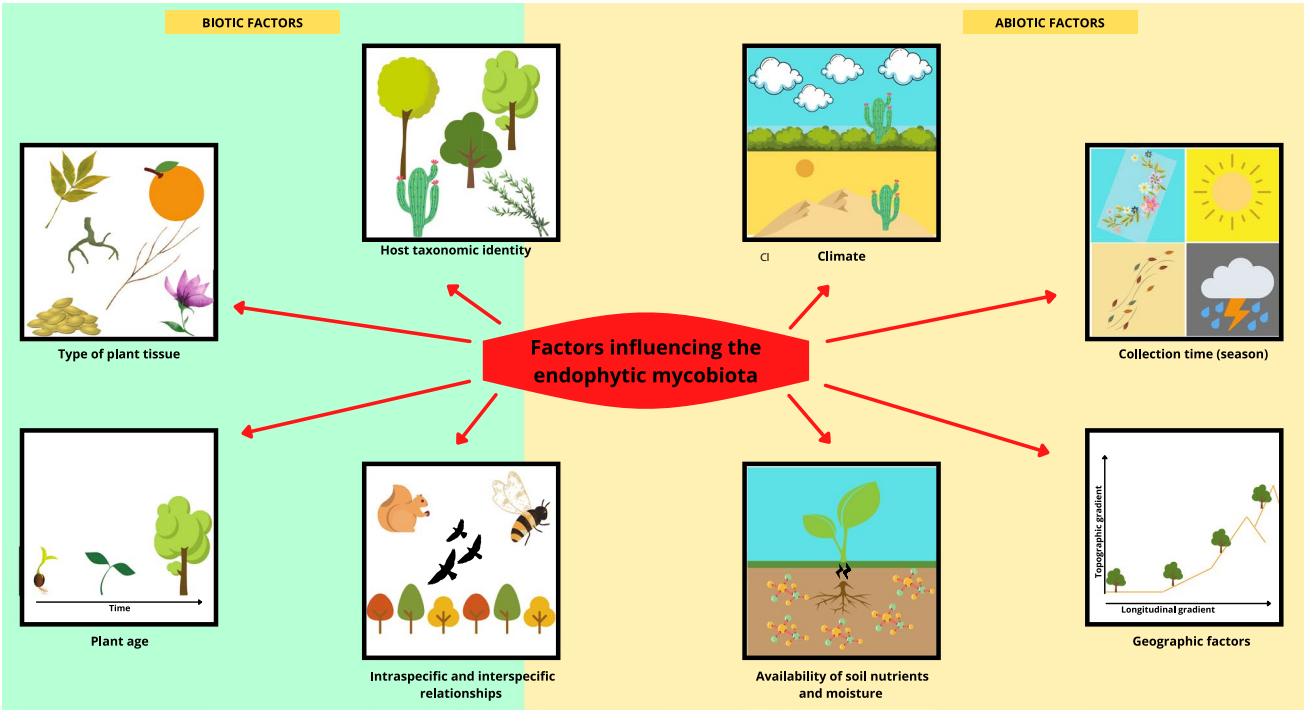


**Fig. 2** Biotic and abiotic factors influencing the assembly of the endophytic mycobiota. The community of endophytic fungi within the same plant species is highly dynamic and the richness and diversity of taxa can be influenced and determined by several biotic and abiotic factors. Image generated based on findings by David et al. (2016), Fernandes et al. (2018), Chi et al. (2019), Yao et al. (2019) and Wu et al. (2020)

rhizoplane and many protocols are available (Table 1). In most cases, they consist of three basic steps: (1) submersion of the tissue in ethanol; (2) immersion of the tissue in the main sterilizing agent, and (3) successive washing with distilled water previously autoclaved. The main sterilizing agents used in rhizoplane disinfection are sodium hypochlorite (NaOCl) (Yao et al. 2019) and mercury chloride ($HgCl_2$) (Du et al. 2020). Other sterilizing agents can also be used such as hydrogen peroxide ($H_2O_2$), paraquat, or 1% peroxyacetic acid ($CH_3COOOH$) in 30% ethanol (Sieber, 2002). According to some authors (Píetro-Souza et al. 2017; Ibrahim et al. 2021), the samples should be washed in running tap water for some times to remove debris, dust, other particles, and main epiphytes (e.g. epiphytic bacteria, yeasts and filamentous fungi) prior to the superficial disinfection of the material to enhance the process.

The physical surface disinfection can be mediated by sonication or ultraviolet (UV) light. In the case of sonication, the plant tissue should be previously washed in tap water and placed in a sonicator or sonication bath containing buffer solution where the sound waves will be propagated, promoting the removal and lysis of microbial cells from the sample surface (Guzmán et al. 2020). Although this method of superficial disinfection is useful for removing epiphytes present in the sample surface (Lundberg et al. 2012), the sound frequency used, application time and tissue characteristics may interfere in the method efficiency. Surface disinfection mediated by UV light also requires that the sample be washed repeatedly in tap water beforehand to remove as much dirt as possible from the plant tissue prior to expose to UV rays for 20 min (Sarsaiya et al. 2020). In general, UV light kills cells by damaging their DNA. However, even the highest-energy UV rays do not have enough energy to penetrate deeply into the plant tissue, so their main effect occurs on the plant tissue surface. Another physical method used to remove epiphytes is plant tissue buckling (Saldierna Guzmán et al. 2020), but this methodology is not widely used due to the various biases associated.

The choice of the disinfection protocol is a critical step to study endophytic microorganisms and the selected methodology should be able to completely remove the epiphytic microbiota without interfering with the endophytic microbiota. Therefore, the chosen method should considered the characteristics of the analyzed tissue/plant species. Burgdorf et al. (2014), for instance, evaluated the effectiveness of two surface disinfection methods, a chemical and a physical methods, on leaves of *Triticum aestivum*. For this, the leaves were artificially sprayed with *Saccharomyces cerevisiae* and then individually submitted to both treatments for the removal of epiphytes. The physical treatment

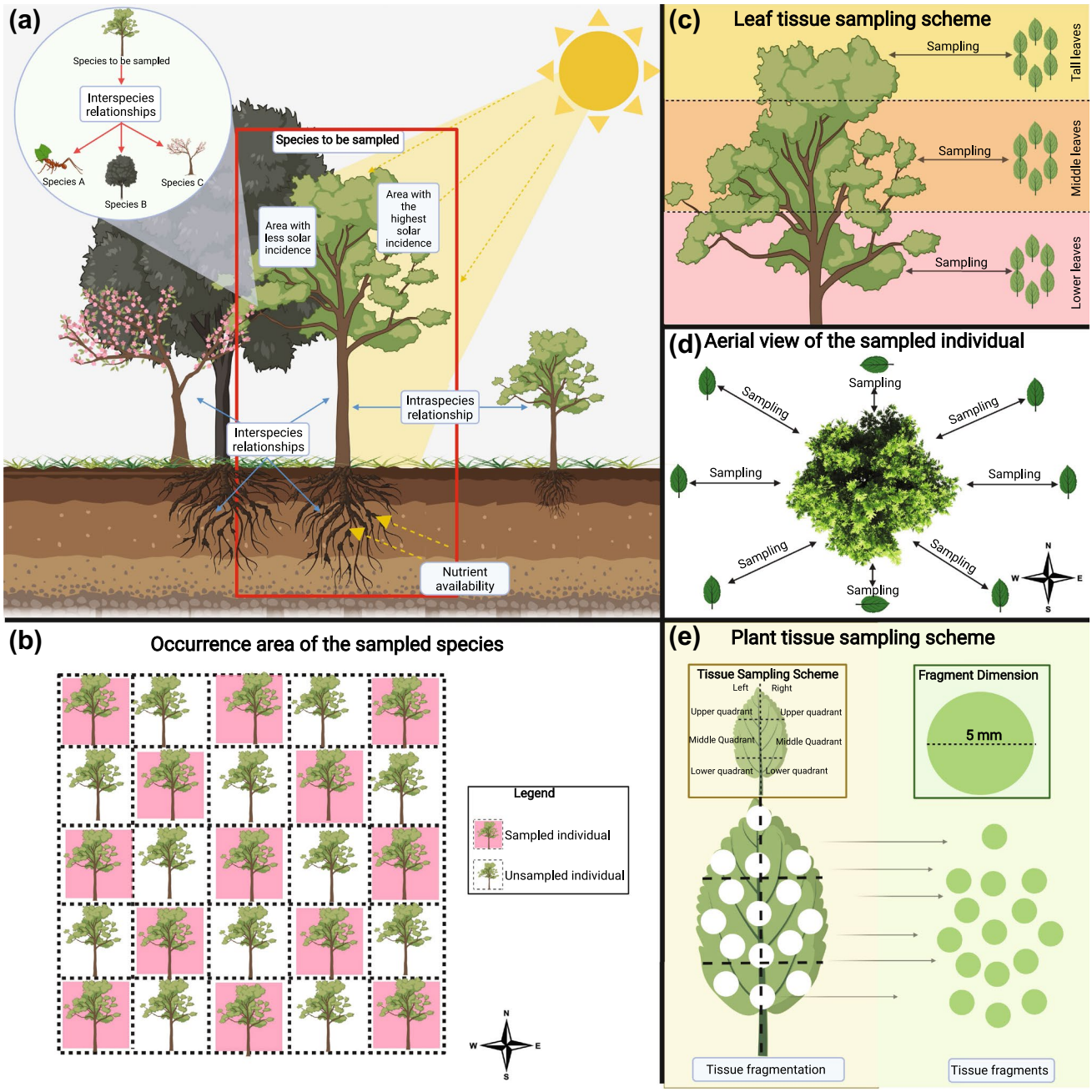


**Fig. 3** Leaf sampling strategy to study endophytic fungi. **a** The plant microbiome is subject to a mosaic of resource availability and environmental conditions that tend to vary on a microenvironmental scale, and are influenced by biotic and abiotic factors. Therefore, these factors should be considered during the sampling process. The red square refers to the specimen to be sampled; the blue and red arrows indicate the influence of biotic factors (inter- and intra-species interactions) that, for example, result in a lower incidence of sunlight in a specific portion of the tree crown to be sampled; the yellow arrows indicate the influence of abiotic factors (e.g. incidence of sunlight, availability of resources). **b** Experimental design of selection of individuals to be sampled to obtain a representative sample. **c** Leaf tissue sampling scheme of the lower, middle and upper leaves. In **d**, the top view of the sampled specimen is shown, and the black arrows indicate that the sampling should be carried out in different regions. In **e**, a tissue fragmentation scheme is presented to obtain a representative sample for the study of endophytic fungi. Image designed from the collection strategy by Wu et al. (2020) and Kluting et al. (2019), Denney et al. (2020), and Hurtado-McCormick et al. (2021) on population variations in microenvironments. Created with BioRender.com

was mediated by sonication for 5 min in a Biosonic sonication bath (Colténe/Whaledent, Altstätten, Switzerland), and successive washings with sterile distilled water. For the chemical treatment, the samples were consecutively immersed in 95% ethanol for 5 s, 0.5% sodium hypochlorite for 2 min, 70% ethanol for 2 min and successive washings

**Table 1** Botanical species, tissue type, methods used for surface disinfection, and culture medium used to study endophytic fungi

| Plant species and botanical family | Tissue | Surface disinfection method | Procedure for validating the effectiveness of surface disinfection | Diameter of the fragment used for recovery of endophytic fungi | Culture medium used for recovery of endophytic fungi | References |
|---|---|---|---|---|---|---|
| *Aegiceras corniculatum* (Myrsinaceae)<br>*Avicennia marina* (Verbenaceae)<br>*Bruguiera gymnorrhiza* (Rhizophoraceae)<br>*Excoecaria agallocha* (Euphorbiaceae)<br>*Kandelia candel* (Rhizophoraceae)<br>*Rhizophora stylosa* (Rhizophoraceae) | Leaves | Consecutive immersion in 75% ethanol for 1 min, 3.25% sodium hypochlorite for 3 min, 75% ethanol for 30 s and three rinses in sterile distilled water | Not informed by authors | Whole leaves | Culture-independent method | Yao et al. (2019) |
| *Securinega suffruticosa* (Euphorbiaceae) | Stem, leaves, and roots | Repeated washing with running tap water to remove dirt and facilitate the surface disinfection process. Surface disinfection was performed as follows: (i) rinse with 75% ethanol for 2–3 min; (ii) washing with sterile water 4–6 times; (iii) superficial disinfection with 0.1% mercury chloride for 3–5 min; and (iv) rinse with sterile water 4–6 times | Fabric printing on Potato Dextrose Agar (PDA) | Fragments with 5 mm × 5 mm | Potato Dextrose Agar with penicillin | Du et al. (2020) |
| *Zanthoxylum bungeanum* (Rutaceae) | Stem, flowers, leaves, fruits, thorns, and roots | Each tissue sampled was disinfected by immersion in 75% ethanol for 2 min, then soaked in 0.2% mercury chloride solution for 10 min, and finally washed three times with autoclaved distilled water | Not informed by authors | Fragments with 1 cm × 1 cm | Potato Dextrose Agar | Li et al. (2016b) |

**Table 1** (continued)

| Plant species and botanical family | Tissue | Surface disinfection method | Procedure for validating the effectiveness of surface disinfection | Diameter of the fragment used for recovery of endophytic fungi | Culture medium used for recovery of endophytic fungi | References |
|---|---|---|---|---|---|---|
| *Sophora tonkinensis* (Fabaceae) | Roots | The root segments were washed in running water for 30 min and rinsed with double-distilled water for 10 min. The samples were sterilized with 75% ethanol for 1 min, 1% sodium hypochlorite for 2 min, 75% ethanol for 30 s, and rinsed three times with sterile double-distilled water | Not informed by authors | Fragments with 1 cm × 1 cm | Potato Dextrose Agar with chloramphenicol | Yao et al. (2017) |
| *Armoracia rusticana* (Brassicaceae) | Roots | The samples were sterilized using commercial bleaching solution diluted four times, followed by washing with sterile distilled water | Printing the sterilized tissue and seeding an aliquot of the last rinse in Sabouraud Dextrose culture medium | Fragments with 1–2 cm | Sabouraud Dextrose Agar (SDA) | Szűcs et al. (2018 |
| *Huperzia serrata* (Lycopodiaceae) | Stem, leaves, and roots | The samples were submerged in 70% ethanol for 1 min, in a sodium hypochlorite solution for 3 min, 2.5% 3% sodium thiosulfate for 5 min, and finally, they were rinsed five times with sterile water | Not informed by authors | Whole leaves | Culture-independent method | Fan et al. (2020) |

**Table 1** (continued)

| Plant species and botanical family | Tissue | Surface disinfection method | Procedure for validating the effectiveness of surface disinfection | Diameter of the fragment used for recovery of endophytic fungi | Culture medium used for recovery of endophytic fungi | References |
|---|---|---|---|---|---|---|
| *Acalypha ornata* (Euphorbiaceae)<br>*Albizia zygia* (Fabaceae)<br>*Alchornea cordifolia* (Euphorbiaceae)<br>*Chrysophyllum albidum* (Sapotaceae)<br>*Ficus exasperata* (Moraceae)<br>*Gomphrena celosioides* (Amaranthaceae)<br>*Millettia thonningii* (Fabaceae)<br>*Newbouldia laevis* (Bignoniaceae) | Leaves | The samples were washed under running tap water to remove dust and debris, followed by consecutive washes with distilled water. Surface disinfection was performed by sequential immersion in 70% ethanol for 60 s, 0.5% sodium hypochlorite for 5 min, 70% ethanol for 30 s, followed by a final rinse in sterilized distilled water for 5 min | Fabric printing on Potato Dextrose Agar | Fragments with 2–4 mm | Potato Dextrose Agar with gentamicin | Ibrahim et al. (2021) |
| *Cymbidium aloifolium* (Orchidaceae) | Leaves, flowers, and roots | Surface sterilization of samples was performed with 0.2% sodium hypochlorite, followed by three washes with sterile distilled water | Fabric printing on Potato Dextrose Agar | Not informed by authors | Potato Dextrose Agar with tetracycline | Chowdappa et al. (2020) |
| *Polygonum acuminatum* (Polygonaceae)<br>*Aeschynomene fluminensis* (Fabaceae) | Roots | The roots were initially washed with neutral detergent (Ype® Neutral Detergent) to remove debris. Surface disinfection was performed with immersion in 70% ethanol for 1 min, 2.5% sodium hypochlorite for 5 min, followed by five rinses in sterile distilled water | Not informed by authors | Fragments with 5 mm×5 mm | Potato Dextrose Agar supplemented with chloramphenicol, streptomycin and tetracycline | Pietro-Souza et al. (2017) |

**Table 1** (continued)

| Plant species and botanical family | Tissue | Surface disinfection method | Procedure for validating the effectiveness of surface disinfection | Diameter of the fragment used for recovery of endophytic fungi | Culture medium used for recovery of endophytic fungi | References |
|---|---|---|---|---|---|---|
| *Cupressus torulosa* (Cupressaceae) | Leaves (*needles*) | The leaf fragments were surface sterilized by consecutive immersion for 1 min in 75% ethanol, followed by immersion in 0.1% mercury chloride for 1 min and several consecutive washing steps with autoclaved distilled water | Sowing aliquots of water from the last rinse in Potato Dextrose Agar | Fragments with 5 mm × 5 mm | Potato Dextrose Agar supplemented with chloramphenicol | Sharma et al. (2016) |
| *Markhamia tomentosa* (Chrysobalanaceae) | Leaves | The plant samples were washed in running water to remove residues and debris. Surface sterilization was performed with sequential immersion in 70% ethanol for 60 s, 10% sodium hypochlorite for 5 min, 70% ethanol for 30 s, followed by a final rinse in sterilized distilled water for 5 min | Not informed by authors | Fragments with 1 cm × 1 cm | Malt Extract Agar (MEA) and Potato Dextrose Agar with streptomycin | Ibrahim et al. (2017) |
| *Catharanthus roseus* (Apocynaceae) | Stem, bark, leaves, and roots | The samples were washed with running water to remove dirt present on the fabrics and then they were washed in a sterilizing solution for two minutes in each solution: 70% ethanol, 0.1% sodium hypochlorite, and finally autoclaved distilled water | Fabric printing on Potato Dextrose Agar | Fragments with 5 mm × 5 mm | Potato Dextrose Agar supplemented with streptomycin | Dhayanithy et al. (2019) |

**Table 1** (continued)

| Plant species and botanical family | Tissue | Surface disinfection method | Procedure for validating the effectiveness of surface disinfection | Diameter of the fragment used for recovery of endophytic fungi | Culture medium used for recovery of endophytic fungi | References |
|---|---|---|---|---|---|---|
| *Mikania cordata* (Asteraceae) | Stems, leaves, branches, and roots | The samples were washed under running water to remove adhered soil particles and other debris. The disinfection procedure was performed in two steps: (i) immersion of samples in 5% sodium hypochlorite for 3 min, followed by washing with sterile distilled water for 1 min (three times); (ii) Immersion of samples in 70% ethanol for 1 min and washing with distilled water for 1 min. The last step was repeated three times | Fabric printing on Potato Dextrose Agar | Fragments with 1 cm × 1 cm | Potato Dextrose Agar supplemented with chloramphenicol | Jayatilake et al. (2020) |
| *Huperzia serrata* (Lycopodiaceae) | Stems, leaves, and roots | The samples were washed with tap water and sequentially sterilized by washing with 75% ethanol for 5 min, 10% sodium hypochlorite for 10 min, and 0.1% mercury chloride for 2 min, and finally they were rinsed with four washes with sterile distilled water | Sowing aliquots of the water from the last rinse in Potato Dextrose Agar | Fragments with 2–5 mm | Potato Dextrose Agar with streptomycin and penicillin | Thi et al. (2019) |
| *Rhizophora mucronata* (Rhizophoraceae) | Leaves | The samples were washed in running water, immersed in 70% ethanol for 2 min, followed by immersion in 4% hypochlorite for 1 min, and rinsed several times in sterile distilled water | Sowing aliquots of the water from the last rinse in Potato Dextrose Agar | Fragments with 2 cm × 2 cm | Medium A (bacteriological agar, malt extract, artificial sea salt, and sterile distilled water) with chloramphenicol | Hamzah et al. (2018) |

**Table 1** (continued)

| Plant species and botanical family | Tissue | Surface disinfection method | Procedure for validating the effectiveness of surface disinfection | Diameter of the fragment used for recovery of endophytic fungi | Culture medium used for recovery of endophytic fungi | References |
|---|---|---|---|---|---|---|
| *Arabidopsis thaliana* (Brassicaceae) | Seeds | The seeds were superficially sterilized by immersion in 70% ethanol with 0.1% Triton-X100 for 1 min, followed by 12 min in 10% A-1 bleach with 0.1% Triton-X100, and three washes in sterile distilled water | Printing the sterilized tissue and seeding an aliquot of the last rinse in different culture medium | Whole leaves | Agar containing Murashige and Skoog (MS) vitamins and sucrose | Lundberg et al. (2012) |
| *Dipsacus asperoides* | Roots | The surface of the plant material was sterilized by immersion in 0.1% mercury chloride for 5 min, then in 75% ethanol for 3 min. Finally, the material was rinsed three times with sterile water | Not informed by authors | Fragments with 5 mm × 5 mm | Potato Dextrose Agar, Tryptone Soy Agar, Tryptone Bovine Extract Agar, and Luria–Bertani culture medium | Gong et al. (2019) |
| *Aegiceras corniculatum* (Myrsinaceae)<br>*Avicennia marina* (Verbenaceae)<br>*Bruguiera gymnorrhiza* (Rhizophoraceae) | Leaves and branches | The segments were surface sterilized by consecutive immersion for 1 min in 75% ethanol, 3 min in 3.25% sodium hypochlorite and 30 s in 75% ethanol | Not informed by authors | Fragments 5 mm long and about 8 mm in diameter | Malt Extract Agar with benzylpenicillin sodium | Li et al. (2016a, b) |

with sterile distilled water. The rinse of the last wash from both procedures was seeded on Potato Dextrose Agar (PDA) medium and analyzed molecularly by PCR (Polymerase Chain Reaction). As result, the authors demonstrated that the physical treatment was the most consistent and effective method for the removal of microorganisms from the surface of host plants compared to the chemical treatment, and more reproducible.

Guzmán et al. (2020) evaluated the effectiveness of four surface disinfection methods in different tissues of two different plant species, namely *Pinus contorta* and *Populus fremontii*. The evaluated protocols consisted of three chemical methods using different sterilizing agents individually (100% ethanol for 1 min; 8.25% sodium hypochlorite for 5 min, and 30% hydrogen peroxide for 1 min) and one physical method consisted of sonication at 40 kHz for 10 min. At the completeness of each procedure, the samples were successively washed with sterile distilled water and the final rinse was recovered. PCR amplification of the final rinses from samples sterilized using the chemical methods gave negative results for all the treatments employed. Moreover, after superficial disinfection, seeding by printing the analyzed tissues in Lysogeny Broth (LB) culture medium, primarily used for bacterial growth, were also negative for all treatments. This showed the efficiency of the chemical treatments employed in removing the epiphytic microbiota. However, scanning electron microscopy (SEM) analysis demonstrated the presence of hyphae, yeast, and bacteria on the leaf surface of both species (*Pinus contorta* and *Populus fremontii*) when sonication (physical method) was employed. In addition, the leaves of *P. fremontii* were not damaged by chemical agents, differently of treatments with hydrogen peroxide and sodium hypochlorite in *P. contorta* leaves, which damage could interfere with the endophytic mycobiome. These results highlight the fact that different plant species require different methodologies to efficiently remove the epiphytic microbiota.

Some authors suggest other protocols of superficial disinfection in addition to those commonly used. For example, Waheeda and Shyam (2017) proposed a disinfection method that includes washing in 3.15% calcium hypochlorite for 10 min, followed by washing in 10% sodium hydrogen carbonate for 15 min, and a final washing in sodium azide 1% for 2 min.

Similar to plant tissue, fungal species are also not equally sensitive to sterilizing agents, and this fact should be considered when surface disinfection methods were chosen. According to Reissinger et al. (2001), ethanol and hypochlorite as well as the exposure time commonly used in surface disinfection protocols are ineffective for the removal and unfeasibility of *Chaetomium globosum* ascospores present in *Hordeum vulgare* leaves. Similarly, Holdenrieder (1989) tested a three-step protocol [(i) immersion in ethanol; (ii) immersion in hypochlorite, and (iii) washing with sterile distilled water] for superficial disinfection of *Picea abies* roots artificially contaminated with conidia of *Penicillium* sp. or *Cylindrocarpon destructans*. The authors demonstrated that the spores of *C. destructans* were eliminated after 60 min, while those of *Penicillium* remained viable for more than two hours. *Penicillium* conidia were only exterminated when hydrogen peroxide was used without prior submersion in ethanol.

The effectiveness of disinfection methods should be essentially evaluated prior when studying endophytic microorganisms, including cheaper or more expensive approaches. This step should be performed properly to validate the adopted procedure. Some authors used the sowing especially in PDA culture medium of the water recovered from the last rinse to validate the effectiveness of disinfection methods (Yao et al. 2017; Szűcs et al. 2018; Fan et al. 2020; Ibrahim et al. 2021). In this case, centrifugation should be used to concentrate the microorganisms present in the last rinse water, followed by the discard of supernatant, and sow of the centrifuged sediment in the culture medium (Sieber 2002). Another alternative is to print the tissue in the culture medium after surface disinfection (Szűcs et al. 2018; Dhayanithy et al. 2019; Jayatilake and Munasinghe 2020.

The effectiveness of the disinfection method can also be verified using DNA-based techniques such as PCR from the water recovered of the last rinse (Burgdorf et al. 2014; Guzmán et al. 2020). This technique is widely used to amplify a segment of DNA of interest. However, depending on the disinfection method employed, false-positive results may occur because the sterilizing agents can promote the cell death of epiphytic microorganisms without degradation of nucleic acids (Burgdorf et al. 2014). The combination of two or more techniques to assess the effectiveness of surface disinfection methods can ensure greater efficiency. The use of scanning electron microscopy (Guzmán et al. 2020) in association with seeding of water from the latter rinsing in culture medium may be an interesting approach.

Thus, the characteristics of the analyzed tissue, the composition of the epiphytic microbiota, the toxicity of the sterilizing agents, and methods used to validate the surface disinfection protocol should be taken into consideration in studies on fungal endophyte communities. Therefore, the performance of previous tests with different sterilization protocols, the previous description of the epiphytic microbiota, and combination of methodologies to validate the surface disinfection method employed are extremely necessary to avoid misinterpretation about the composition of the culturable endophytic mycobiota.

## Culture medium for growth of endophytic fungi

There are many culture medium protocols available to recover endophytic fungi, with PDA as the most frequently used (Table 1). Other growth media such as Malt Extract Agar (Li et al. 2016a, b), Agar containing Murashige and Skoog (MS) vitamins and sucrose (Lundberg et al. 2012), Hagem Minimal Medium (Khan et al. 2016), Czapek medium (Chand et al. 2020), Tryptone Soybean Agar, Tryptone Bovine Extract Agar, and Luria–Bertani medium (Gong et al. 2019), which is also known as LB broth, Lysogeny broth or Luria broth, can be efficiently used to recover endophytic fungi from plant samples. Although several culture media are used for recovery and cultivation of endophytic fungi, they must have a slightly acidic pH range (5.8–6.0 pH) (Lundberg et al. 2012; Li et al. 2016a, b; An et al. 2020). Depending on the purpose of the study and the characteristics of the habitat (i.e. host plant species), the medium commonly used can be nutritionally supplemented and/or replaced by specific medium to better achieve the objectives of the study (Pietro-Souza et al. 2017; Hamzah et al. 2018; Hamzah et al. al. 2018).

In the case of a study focused on description of the diversity of easily cultivable endophytic fungi, a single culture medium such as PDA (Ibrahim et al. 2021) can be used. To increase the number of retrieved taxa, two or more different culture media can be employed in addition to PDA (Man et al. 2015; Gong et al. 2019). Supplementation of culture medium with plant material from the host species can also be used to improve the recovery of endophytic fungi (Mourad et al. 2018). In this case, the plant material should be properly processed and autoclaved in order to avoid contamination. The use of a water agar medium is also a great option to be considered in studies on diversity of fungal endophyte, since this medium is poor in nutrients and prevents fast-growing fungal species from standing out over slower growing ones (Stone et al. 2004).

Last but not least, the use of antibiotics to inhibit endophytic bacteria is essential in studies on diversity of cultivable fungal endophytes (Yao et al. 2017). The use of one or more broad-spectrum antibiotics such as sodium benzylpenicillin (Li et al. 2016a, b), chloramphenicol (Hamzah et al. 2018), streptomycin (Ibrahim et al. 2016), gentamicin (Ibrahim et al. 2021) and tetracycline (Chowdappa et al. 2020) is strongly recommended.

## Cultivation conditions, isolation and culture purification

According to the objectives of the study, the cultivation conditions of endophytic fungi in artificial culture medium are diverse. The temperature commonly used for incubation of Petri dishes usually ranges from 25 ºC (Li et al. 2016a, b; Du et al. 2020) to 28 ºC (Yao et al. 2017), with period of incubation between 3 and 20 days (Li et al. 2016a, b; Yao et al. 2017; Du et al. 2020), which can be extended for up to 6 weeks if necessary (Yao et al. 2017). In the case of cultures that require a longer period of incubation or are kept under environmental conditions outside greenhouses or BOD incubators (Bio-Oxygen Demand), it is necessary to seal the plates with Parafilm to minimize risks of contamination and/or drying of the culture medium (Szűcs et al. 2018; Ababutain 2021). Cultivation can also be carried out with or without incidence of light (Duan et al. 2019; Agbessenou et al. 2020). In this sense, the endogenous circadian clocks present in both prokaryotes and eukaryotes provide the machinery by which they keep in synchrony with the external environment. In fungi, the clock has been shown to control daily rhythms (Dunlap and Loros 2017) in spore development and liberation, supporting their survival.

Plates seeded with fragmented plant tissue should be monitored daily. Fragmentation of plant tissue can be achieved with the aid of a scalpel, eyelet pliers or scissors, then printed on a culture medium and incubated. Although fragments with 5 mm × 5 mm are commonly used, other dimensions have also been employed (Table 1). As fungal growth frequently occurs from the edges of the inoculated plant tissue, the isolation is recommended by collecting the hyphae from the edges of the fungal colonies, followed by seeding in fresh culture medium with or without antibiotics (Li et al. 2016a, b; Ibrahim et al. 2017; Yao et al. 2017; Ababutain 2021). This step should be repeated several times until a monoculture of a endophytic fungus strain is reached with a uniform colony (Ibrahim et al. 2017). Then, the monosporic and/or hypha tip purification must be performed (Supaphon et al. 2013; Duan et al. 2019) for later deposit in a mycological culture collection and/or molecular identification of the isolates.

## Morphological or morphotype grouping, and storage of endophytic fungi colonies

After the purification process, the fungal isolates are grouped based on their macro- and micromorphological characteristics (Dhayanithy et al. 2019; Jayatilake and Munasinghe 2020). For this, macroscopic vegetative characteristics that includes color, texture, topography, and diffuse pigmentation of structures, and color and topography of the colony's dorsum, are considered, and also analyses of their microscopic reproductive structures, using microculture and/or sporulation methods (Dos Banhos et al. 2014). The macroscopic classification allows grouping the isolates into morphotypes, while the microscopic classification allows the morphological classification of the isolates. However, the arrangement

in morphotypes and/or morphological features does not reflect the real phylogeny of the organisms, even more considering non-sporulating fungal endophyte species (Li et al. 2016a, b; Yao et al. 2017; Du et al. 2020) that requires robust tools such as molecular methods to achieve the taxonomic classification of the isolates.

To avoid losses of isolated colonies caused by contamination, re-isolation on PDA prepared inclined cryotubes and subsequent storage in a cold chamber at 4° C and freezer – 80° C is recommended (Li et al. 2016b; Wang et al. 2019). For long-term preservation of cultures, mycelia and spores should be transferred to 20% glycerol in ultrapure distilled water and stored at − 80° C (Wang et al. 2019). Another alternative for long-term preservation of monocultures is to transfer small pieces of pre-inoculated culture medium to sterile microtube containing 30% glycerol (v/v) and sterile rice medium, followed by incubation at 25 °C. After a fungal growth is observed, the microtubes should be transferred to a cold room (4 °C), and storage in a freezer at − 20 °C, respectively (Ibrahim et al. 2017).

## Molecular identification of isolated endophytic fungi

Despite the development of different methods to promote fungal sporulation (Taylor et al. 1999; Sun et al. 2008a), more than 50% of endophytic fungal isolates do not sporulate in cultures (*Mycelia sterilia*) (Wang 2008; Wang and e Guo LD 2007; Sun et al. 2008b, 2011), which makes the conventional classification based on observation of the reproductive structures of fungi impossible. Furthermore, even for those isolates capable of sporulating, a polyphasic approach that includes molecular analysis plus observation of reproductive structures is strongly recommended to achieve a precise fungi identification (Sun and Guo 2012).

There are many protocols used for molecular characterization of endophytic fungal isolates (Table 2). Briefly, this approach requires the following steps: (I) genomic DNA (gDNA) extraction; (II) PCR amplification of conserved DNA sequences (rDNA) using specific or universal oligonucleotide primers forward and reverse; (III) sequencing of PCR products; (IV) sequence processing and comparison with related sequences deposited in DNA databases; (V) data interpretation and phylogenetic tree reconstruction (Hamzah et al. 2018; Thi et al. 2019; Jayatilake and Munasinghe 2020). In addition, DNA cloning using cloning vectors can also be employed prior to sequencing (Atsatt and Whiteside 2014).

DNA extraction is a critical step to achieve the isolate characterization and should be carried out from monocultures well established previously (Li et al. 2016a, b; Wang et al. 2019). In general, it begins with the fungal cell lysis by maceration in liquid nitrogen (Li et al. 2016a, b) or cell lysis kits (Ababutain 2021). After cell disintegration, several homemade protocols such as the standard phenol–chloroform method (Ibrahim et al. 2017; Tang et al. 2020) and kits commercially available (Du et al. 2020; Ababutin 2021) can be employed. To avoid biases, it is strongly recommended the utilization of commercial kits according to the manufacturer's instructions (Ababutin 2021). However, they can be adapted eventually to achieve better results (Du et al. 2020) when necessary. Subsequently, the extracted DNA should be purified, recovered and analyzed on 1% agarose gels after electrophoresis in 1 × TBE running buffer, for instance, and storage at − 20° C. The extracted DNA quality and integrity should be ensured prior to further analyses (Ibrahim et al. 2017; Tang et al. 2020).

PCR amplification of the extracted genomic DNA targeting specific DNA regions (Barth Reller et al. 2007) can be performed using different PCR conditions as shown in Table 2. The PCR technique is still widely used to study endophytic fungi from monocultures prior to sequencing, generating PCR products (i.e. amplicons) of target sequences. PCR reactions require PCR buffers, cofactors, deoxynucleotides (dNTPs—DNA building blocks), specific primers to the DNA target region (e.g. ITS and 18 rDNA), thermostable DNA polymerase, and the genomic DNA as template (Table 2). All components should be in a proper concentration following the manufacturer's instructions. The DNA amplification is performed in a thermocycler through numerous cycles of temperature variation. Briefly, PCR thermal cycling conditions comprise three steps basically: double-stranded DNA denaturation, primer annealing, and extension/elongation (Chowdappa et al. 2020; Ibrahim et al. 2021) (Table 2). It is important to emphasize that the annealing temperature depends on the primers design (Sarsaiya et al. 2020), especially concerning the GC content. Finally, the amplicons obtained are purified and visualized on gel electrophoresis to confirm the PCR amplification, and then subjected to sequencing (Ibrahim et al. 2017; Tang et al. 2020; Du et al. 2020).

Despite advances in DNA sequencing technologies, sequencing of amplicons from fungal endophyte using Sanger sequencing is still the method largely used (Li et al. 2016a, b; Ibrahim et al. 2017; Yu et al. 2018; Du et al. 2020; An et al. 2020; Tang et al. 2020; Ababutain 2021). After sequencing completion, the nucleotide sequences obtained are treated in appropriate softwares for noise removal and quality scores (Phred > 20). Then, the newly digital sequences are submitted to DNA public databases, and compared with previous deposited sequences for identification (Ibrahim et al. 2017; Tang et al. 2020; Du et al. 2020). Pairwise comparisons are usually carried out using BLASTn tool (Basic Local Alignment Search Tool) from the NCBI (National Center for Biotechnology Information) (Li et al.

**Table 2** Main steps and PCR protocols for molecular detection of endophytic fungi using culture-dependent methods

| DNA extraction protocol | Primers used for PCR amplification | Amplified genomic region | Reagents used in PCR | Amplification conditions | Amplicon size | Database used to search for sequence similarity | References |
|---|---|---|---|---|---|---|---|
| Genomic DNA extraction was performed using the CTAB-based method following the kit instructions of Biotech Bioengineering Co. Ltd | ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGA TATGC-3′) | Internal transcribed spacer region (ITS) | ■12.5 µL of 2×Pfu PCR Mix;<br>■0.5 µL of ITS1;<br>■0.5 µL of ITS4;<br>■1.5 µL of DNA;<br>■10 µL of ultrapure water | Initial denaturation step at 94 °C for 5 min; followed by 35 cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 30 s; and final step at 72 °C for 15 min | 400–800 bp | Genbank | Du et al. (2020) |
| The mycelia were macerated using liquid nitrogen and autoclaved mortar, then DNA extraction was performed using the TaKaRa MiniBEST plant genomic DNA extraction kit (Takara Biotechnology Co., Ltd., Dalian, China, Code No. 9768) | ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) | Internal transcribed spacer region (ITS) | ■15 µL of Premix TaqTM (Takara Biotechnology Co., Ltd., Dalian, China No. RR003A);<br>■0.5 µL of DNA;<br>■1.0 µL of primer ITS1;<br>■1 µL of primer ITS4;<br>■12.5 µL of ultrapure water | Initial denaturation step at 94 °C for 3 min, followed by 34 cycles of 30 s at 94 °C, 30 s of primer annealing at 54 °C, 45 s of extension at 72 °C, and final elongation step of 10 min at 72 °C | Not informed by authors | Genbank | Li et al. (2016a, b) |
| The mycelia were previously macerated in liquid nitrogen, and then TaKaRa MiniBEST bacterial genomic DNA extraction kit (Dalian, China) was used for DNA extraction | ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) | Internal transcribed spacer region (ITS) | ■5.0 µL of 10×Taq buffer;<br>■4.0 µL of 200 mmol/L dNTPs; 2.0 µL of ITS1 at 10 µM;<br>■2.0 µL of ITS4 at 10 µM;<br>■0.5 µL Ex Taq enzyme (TaKaRa, Dalian);<br>■5.0 µL of genomic DNA | Initial denaturation step at 95 °C for 1 min, followed by 35 cycles of 15 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C; and a final extension of 10 min at 72 °C | Not informed by authors | Genbank | An et al. (2020) |
| Fungal genomic DNA was extracted using the SDS method | ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) | Internal transcribed spacer region (ITS) | Not informed by authors | Not informed by authors | 500–750 bp | Genbank | Tang et al. (2020) |

**Table 2** (continued)

| DNA extraction protocol | Primers used for PCR amplification | Amplified genomic region | Reagents used in PCR | Amplification conditions | Amplicon size | Database used to search for sequence similarity | References |
|---|---|---|---|---|---|---|---|
| The adopted DNA extraction procedure was the standard phenol–chloroform method with modifications | ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) | Internal transcribed spacer region (ITS) | ■2.5 µL of 10×PCR buffer;<br>■0.5 µL of 1 mmol dNTPs<br>■2.5 µL of 10 pmol ITS1<br>■2.5 µL of 10 pmol ITS4<br>■1 µL of 40 ng of DNA<br>■0.25 µL of 5 units of Taq polymerase | Initial denaturation step at 94 °C for 2 min; 35 cycles of 94 °C for 1 min, primer specific annealing temperature at 57 °C for 1.30 min and extension at 72 °C for 2 min; and a final extension at 72 °C for 4 min | 400–750 bp | Genbank | Ibrahim et al. (2017) |
| Genomic DNA was extracted using a fungal genomic DNA extraction kit (Sangon, Shanghai, China) | ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) | Internal transcribed spacer region (ITS) | ■5 µL of 10×PCR buffer (10 mM);<br>■1 µL of dNTP mix (10 mM);<br>■0.5 µL of Taq polymerase (5 U/µL);<br>■2 µL of each primer (10 mM)<br>■5 ng of DNA template;<br>■Double distilled water | Initial denaturation step at 94 °C for 5 min; followed by 35 cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 30 s; and finally 72 °C for 5 min | Not informed by authors | Genbank | Yu et al. (2018) |
| Genomic DNA was extracted from endophytic fungi samples using the Genomic DNA purification kit (Promega, USA) | 18S rRNA forward (5′-GCTTAATTTGACTCAACACGGGA-3′) and rRNA 18S reverse (5′-AGCTATCAATCTGTCAATCCTGTC-3’) | 18S rDNA | Not informed by authors | Initial denaturation step at 95 °C for 10 min, followed by 35 cycles of 95 °C for 1 min, 67.7 °C for 1 min and 15 s and 72 °C for 2 min, and finally an extension step at 72 °C for 5 min | Not informed by authors | FungiDB | Ababutain et al. (2021) |

2016a, b; Ibrahim et al. 2017; Yu et al. 2018; Du et al. 2020; An et al. 2020; Tang et al. 2020). Sequence similarity of 94% allows classification at the genus level and > 97% at the species level for constitutive gene (Li et al. 2016a, b). Other bioinformatics tools and databases can also be employed for the taxonomic identification of endophytic fungi (Ababutin 2021). Nevertheless, fungal identification at the species level is hardly achieved using a single DNA region (Noriler et al. 2018) due to highly conserved sequences. Thus, analysis of DNA barcodes with hypervariable regions may improve the identification of endophytic fungi at the species level.

Finally, the taxonomic position of fungal isolates can be accessed by phylogenetic tree reconstructions. Phylogenetic analysis may extend the knowledge on geographic occurrence of the isolates and host plants especially in studies on diversity of endophytic fungi. Some tools to assess the phylogenetic relationships among endophytic fungi taxa can be found in Li et al. (2016a, b) and An et al. (2020).

## Barcodes for identification of endophytic fungi and related primers

The nuclear ribosomal internal transcribed spacer (ITS) region is the most used DNA molecular marker employed to identify fungi (Schoch et al. 2012; Koljalg et al. 2013) (Fig. 4). Coupled with the 18S rRNA and 28S rRNA gene sequences, the ITS region is present in several copies in the fungal genome and other eukaryotes. Moreover, the ITS region is considered as a primary DNA barcode region for identification of fungi taxa (Kiss 2012). Barcode sequences are short universal and standardized DNA sequences easily PCR amplified (450–800 bp), and commonly found in a taxonomic group, but highly divergent at the species level, which enables accurate and rapid identification (Blackwell 2011). These sequences should be unique and stable within each species (Schoch et al. 2012). To be considered a good barcode, the interspecific variation must exceed the intraspecific variation to allow distribution of distances among different taxa (Hebert et al. 2003). As the ITS region is largely employed as a primary barcode region for fungal identification, several universal oligonucleotide primers have been designed (Lücking et al. 2020) (Table 3). The average size of amplicons generated by PCR amplification from this region either for studies using Sanger sequencing or high-performance sequencing generally ranges from 450 to 800 bp (Li et al. 2016a, b; Ibrahim et al. 2017; Yu et al. 2018; Du et al. 2020; An et al. 2020).

According to Schoch et al. (2012), the ITS region quite supplies the requirements for fungal species discrimination in the vast majority of cases. When associated with DNA sequencing for molecular identification of endophytic fungi, the ITS region is undoubtedly a good choice as a DNA barcode (Li et al. 2016a, b; Ibrahim et al. 2017; Yu et al. 2018; Du et al. 2020; An et al. 2020; Tang et al. 2020). However, this region may result in low resolution for fungal species-level differentiation (Noriler et al. 2018) particularly for the taxonomic classification of *Aspergillus* spp., *Alternaria* spp., *Cladosporium* spp., *Colletotrichum* spp., *Fusarium* spp. and *Penicillium* spp. (Lücking et al. 2020). Analyses of the ITS sequence accuracy showed that for 6 and 17% of yeast and filamentous fungi species, respectively, the classification at a species level was not possible from ITS as a barcode region (Vu et al. 2016, 2019). Furthermore, within the genome of a single fungal cell there may be numberless copies of ITS sequences (Kiss 2012), with intergenomic variability (Smith et al. 2007; Weitemier et al. 2015). Such variations can interfere with the PCR amplification (Xu, 2016) due to primer

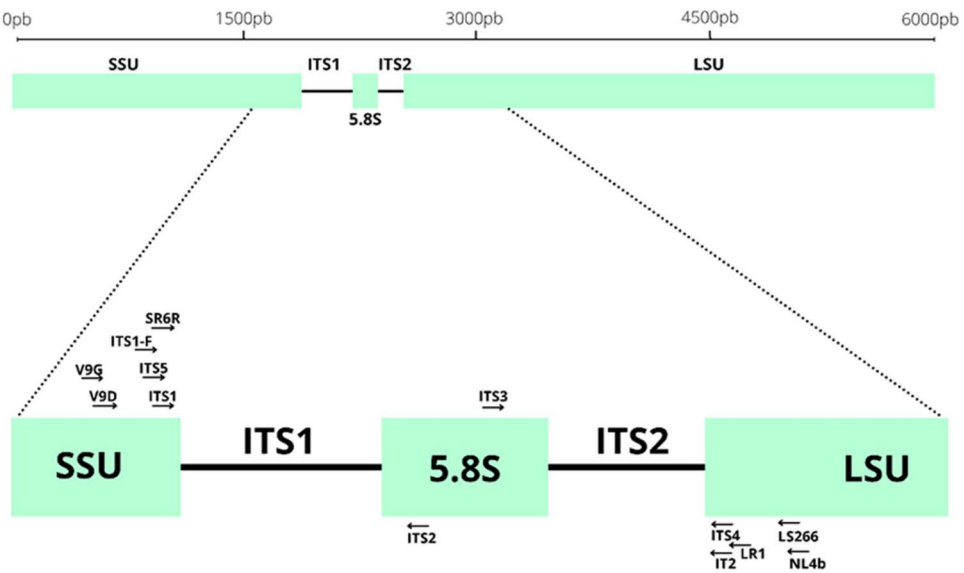


**Fig. 4** Ribosomal RNA nuclear genes and ITS regions. The ITS region includes the ITS1 and ITS2 sequences separated by the 5.8S rRNA gene, which are located between the 18S rRNA (SSU) and 28S rRNA (LSU) genes (Blackwell et al. 2011). The small arrows observed from the ITS region magnification demonstrate the annealing position of primers used for PCR amplification from this region (White et al. 1990; Gardes and Hester 1993; O'Donnell, 1993; Gerrits van den Ende and de Hoog, 1999; Beguin et al. 2012)

**Table 3** Oligonucleotide primers for universal fungal barcodes to taxonomic identification

| Barcode | Primer name | Primer sequence | Target gene loci | Amplicon length (bp)[a] | References |
|---|---|---|---|---|---|
| Primary barcode | ITS1 | 5′-TCCGTAGGTGAA CCTGCGG-3′ | ITS1-5.8S-ITS2 rDNA | 500–800 | White et al. (1990) |
| Primary barcode | ITS2 | 5′-GCTGCGTTCTTC ATCGATGC-3′ | ITS1-5.8S-ITS2 rDNA | 500–800 | White et al. (1990) |
| Primary barcode | ITS3 | 5′-GCATCGATGAAG AACGCAGC-3′ | ITS1-5.8S-ITS2 rDNA | 500–800 | White et al. (1990) |
| Primary barcode | ITS4 | 5′-TCCTCCGCTTATTGA TATGC-3′ | ITS1-5.8S-ITS2 rDNA | 500–800 | White et al. (1990) |
| Primary barcode | ITS5 | 5′-GAAGTAAAAGTC GTAACAAGG-3′ | ITS1-5.8S-ITS2 rDNA | 500–800 | White et al. (1990) |
| Primary barcode | ITS1-F | 5′-CTTGGTCATTTA GAGGAAGTAA-3′ | ITS1-5.8S-ITS2 rDNA | Variable | Gardes and Hester (1993) |
| Primary barcode | ITS4-B | 5′-CAGGAGACTTGT ACACGGTCCAG-3′ | ITS1-5.8S-ITS2 rDNA | Variable | Gardes and Hester (1993) |
| Primary barcode | IT2 | 5′-CCTCCGCTTATTGAT ATGCTTAGG-3′ | ITS1-5.8S-ITS2 rDNA | 300–650 | Beguin et al. (2012) |
| Primary barcode | NL4b | 5′-GGATTCTCACCC TCTATGAC-3′ | ITS1-5.8S-ITS2 rDNA | 300–700 | O'Donnell (1993) |
| Primary barcode | SR6R | 5′-AAGTATAAGTCG TAACAAGG-3′ | ITS1-5.8S-ITS2 rDNA | Variable | Vilgalys and Hester (1990) |
| Primary barcode | LR1 | 5′-GGTTGGTTTCTT TCCT-3′ | ITS1-5.8S-ITS2 rDNA | ~400 | Vilgalys and Hester (1990) |
| Primary barcode | V9D | 5′-TTAAGTCCCTGC CCTTTGTA-3′ | ITS1-5.8S-ITS2 rDNA | > 1000 | Gerrits van den Ende and de Hoog (1999) |
| Primary barcode | V9G | 5′-TACGTCCCTGCC CTTTGTA-3′ | ITS1-5.8S-ITS2 rDNA | > 1000 | Gerrits van den Ende and de Hoog (1999) |
| Primary barcode | LS266 | 5′-GCATTCCCAAAC AACTCGACTC-3′ | ITS1-5.8S-ITS2 rDNA | > 1000 | Masclaux et al. (1995) |
| Primary barcode | 18S rRNA F | 5′-GCTTAATTTGAC TCAACACGGGA-3′ | 18S rDNA (SSU) | ~1000–1500 | Ababutain et al. (2021) |
| Primary barcode | 18S rRNA R | 5′-AG-AGCTATCAATCT GTCAATCCTGTC-3′ | 18S rDNA (SSU) | ~1000–1500 | Ababutain et al. (2021) |
| Primary barcode | NS1 | 5′-GTAGTCATATGC TTGTCTC-3′ | 18S rDNA (SSU) | ~600 | White et al. (1990) |
| Primary barcode | NS4 | 5′-CTTCCGTCAATT CCTTTAAG-3′ | 18S rDNA (SSU) | ~600 | White et al. (1990) |
| Primary barcode | LROR | 5′-ACCCGCTGAACT TAAGC-3′ | 28S rDNA (LSU) | > 1000 | Vilgalys and Sun (1994) |
| Primary barcode | LR5 | 5′-TCCTGAGGGAAA CTTCG-3′ | 28S rDNA (LSU) | > 1000 | Vilgalys and Sun, (1994 |
| Primary barcode | EF1-1002F | 5′-TTCATCAAGAAC ATGAT-3′ | TEF1α | ~500 | Stielow et al. (2015) |
| Primary barcode | EF1-1018F | 5′-GAYTTCATCAAG AACATGAT-3′ | TEF1α | ~500 | Stielow et al, (2015) |
| Secondary barcode | EF1-1620R | 5′-GACGTTGAADCCR ACRTTGTC-3′ | TEF1α | ~500 | Stielow et al, (2015) |
| Secondary barcode | EF1-1688R | 5′-GCTATCATCACA ATGGACGTTCTT GGAG-3′ | TEF1α | ~500 | Stielow et al. (2015) |
| Secondary barcode | EF1 | 5′-ATGGGTAAGGA (A/G) GACAAGAC-3′ | TEF1$\alpha$ | ~500–600 | O'Donnell et al. (1998) |
| Secondary barcode | EF2 | 5′-GGA (G/A) GTACCA GT (G/C) ATCATG TT-3′ | TEF1$\alpha$ | ~500–600 | O'Donnell et al. (1998 |

**Table 3** (continued)

| Barcode | Primer name | Primer sequence | Target gene loci | Amplicon length (bp)[a] | References |
|---|---|---|---|---|---|
| Secondary barcode | NL11 | 5′-CTGAACGCCTCT AAGTCAG-3′ | IGS | ~700 | Aoki et al. (2003) |
| Secondary barcode | CNS1 | 5′-GAGACAAGCATA TGACTAC-3′ | IGS | ~700 | Aoki et al. (2003) |
| Secondary barcode | Bt 2 a | 5′-GGTAACCAAATC GGTGCTGCTTTC-3′ | β-Tubulin II | ~550 | Glass and Donaldson (1995) |
| Secondary barcode | Bt 2 b | 5′-ACCCTCAGTGTA GTGACCCTTGGC-3′ | β-Tubulin II | ~550 | Glass and Donaldson (1995) |
| Secondary barcode | CMD5 | 5′-CCGAGTACAAGG ARGCCTTC-3′ | Calmodulin (CaM) | ~580 | Hong et al. (2005) |
| Secondary barcode | CMD6 | 5′-CCGATRGAGGTC ATRACGTGG 3 ' | Calmodulin (CaM) | ~580 | Hong et al. (2005) |
| Secondary barcode | CF1 | 5′-GCCGACTCTTTG ACYGARGAR-3′ | Calmodulin (CaM) | ~500 | Peterson et al. (2005) |
| Secondary barcode | CF4 | 5′-TTTYTGCATRAG YTGGAC-3′ | Calmodulin (CaM) | ~500 | Peterson et al. (2005) |
| Secondary barcode | PenF2 | 5'-TWAGTTTCTGAT TATTAGTACCTA GTTT-3′ | Cox 1 | ~600 | Seifert et al. (2007) |
| Secondary barcode | PenR2 | 5′-AAACTAGGTACT AATAATCAGAAA CTWA-3′ | Cox 1 | ~600 | Seifert et al. (2007) |
| Secondary barcode | Cox2-F | 5'-GGCAAATGGGTT TTCAAGATCC-3′ | Cox 2 | ~600 | Hudspeth et al. (2000) |
| Secondary barcode | Cox2-R | 5'-CCATGATTAATA CCACAAATTTCA CTAC-3′ | Cox 2 | ~600 | Hudspeth et al. (2000) |
| Secondary barcode | 5F | 5′-GAYGAYMGWGATC AYTTYGG-3′ | RNA polymerase II (RPB2) | ~1000 | Liu et al. (1999) |
| Secondary barcode | 7CR | 5′-CCCATRGCTTGY TTRCCCAT-3′ | RNA polymerase II (RPB2) | ~1000 | Liu et al. (1999) |
| Secondary barcode | 5Feur | 5′-GAYGAYCGKGAY CAYTTCGG-3′ | RNA polymerase II (RPB2) | ~1000 | Houbraken et al. (2012) |
| Secondary barcode | 7CReur | 5′-CCCATRGCYTGY TTRCCCAT-3′ | RNA polymerase II (RPB2) | ~1000 | Houbraken et al. (2012) |

*SSU* small subunit, *LSU* large subunit

[a]Although the estimated amplicon sizes in base pairs (bp) are provided in column five, it should be considered that this value may vary upwards or downwards depending on the phylogenetic group of fungi studied

mismatching, jeopardizing the study. Therefore, in cases where precise identification is not possible through the ITS region, other DNA regions can be employed to allow a reliable identification (Lücking et al. 2020). In these situations, the use of secondary barcodes is strongly recommended to achieve a better discrimination among taxa. The D1/D2 regions, for instance, are well employed for species-level classification of yeasts (Scorzetti et al. 2002).

In addition, protein-coding genes employed as secondary barcodes are useful to identify fungal species. Some authors have reported their efficacy over the ITS region (Frisvad et al. 2004; Seifert 2009; Sarmiento-Ramirez et al. 2014). For the Ascomycota phylum, these genes are considered better than rRNA genes to solve close relationships at different taxonomic and phylogenetic levels (Schoch et al. 2009). Among the protein-coding genes, the β-tubulin II gene (TUB2), cytochrome c oxidase subunit I (COX1) and subunit II (COX2), DNA-directed RNA polymerase II largest subunit (RPB1) and second largest subunit (RPB2), translational elongation factor 1α (TEF1), DNA topoisomerase I (TOP1), and phosphoglycerate kinase (PGK) have been proposed (Lücking et al. 2020; Crous et al. 2020). To classify *Penicillium* spp. and *Aspergillus* spp. at the species level, the use of β-tubulin was reported by Frisvad et al. (2004) and Samson et al. (2014), respectively. Similarly, Short et al. (2013) employed the TEF1 sequence-based diversity for *Fusarium* spp.

The oligonucleotide primers available for PCR amplification of primary barcode regions should be specific and capable of amplifying a sufficient sequence length for further analyses (Lücking et al. 2020). The ITS1, ITS2, and ITS4 primers are commonly used to reach this goal because they produce amplicons of 400–800 bp (Li et al. 2016a, b; Ibrahim et al. 2017; Yu et al. 2018; An et al. 2020; Du et al. 2020; Tang et al. 2020). Other specific ITS primers were designed to avoid cross-reactions with animal and/or plant DNA such as ITS1F (Gardes and Hester 1993), IT2 (Beguin et al. 2012), NL4b (O'Donnell 1993), V9D, V9G and LS266 (Gerrits van den Ende and de Hoog 1999). The primers designed for secondary barcode regions should be universal and able to amplify a reliable region to allow the fungal strain identification when primary barcode regions possess insufficient resolution (Frisvad et al. 2004; Stielow et al. 2015). The EF1-1018F (Al33F)/EF1-1620R (Al33R) and EF1-1002F (Al34F)/EF1-1688R (Al34R) primer sets designed for TEF1α (Stielow et al. 2015) are good examples. Some primer sets designed for universal fungal barcodes to different loci are summarized in Table 3.

## Secondary structure prediction of the ITS rRNA region and molecular phylogeny: an integrated approach for a better speciation of endophytic fungi

Novel species of non-sporulating fungi of the genus *Muscodor* with bio-fumigant and bio-preservative activities have been successfully identified using ITS sequences (Kapoor et al. 2018). For this, the secondary structural prediction of the ITS1, 5.8S, and ITS2 regions presented a vital accuracy for fungal speciation. In addition, the ITS hypervariable nature hinders the design of stable phylogenetic trees (Kapoor et al. 2018). Therefore, the ITS structural analysis along with the ITS primary sequence information have been provided new insights for identification of fungal species.

As ITS sequence is a popular primary fungal barcode marker, the nuclear ribosomal internal transcribed spacer 1 (ITS1) region has used in studies on anaerobic fungi (phylum Neocallimastigomycota) in particular. Due to problems involving the reconstruction of stable phylogenetic trees, Koetschan et al. (2014) proposed a common core secondary structure of ITS1 from anaerobic fungi employing a Hidden Markov Model-based ITS1 sequence annotation and a helix-wise folding approach. The authors integrated the additional structural information into phylogenetic analyses and presented an automated sequence-structure-based taxonomy of ITS1 to anaerobic fungi. According to the authors, this methodology is possible to be applied to other fungal groups, probably including endophytic fungi.

In addition, exploring the secondary structure of the nuclear ribosomal internal transcribed spacer 2 (ITS2) has been a promising approach in fungal species delimitation. Sundaresan et al. (2019) investigated the presence of insertion/deletion (INDELs) and substitutions in fungal delineation at species level by the analysis of ITS2 molecular morphometrics effectiveness in species delimitation of Ascomycota. As result, the authors reported that the ITS2 molecular morphometric analysis is an efficient third dimensional study of fungal species boundaries, which may help to alleviate the challenges in molecular characterization.

An endophytic fungus with considerable amount of antimicrobial activity isolated from healthy leaf tissues of *Houttuynia cordata* Thunb., an ethnomedicinal plant of North East India, was previously identified as *Colletotrichum* sp. and then characterized by its genomic ITS rDNA and ITS2 sequences (Talukdar et al. 2021). According to phylogenetic analyses, the isolate was clustered with *Colletotrichum coccodes*. However, to confirm the endophytic lifestyle of the isolate, the ITS2 RNA secondary structure study was undertaken, and showed differences in the folding pattern as well as in motif structures compared to those of pathogenic *C. coccodes*, since species of *Colletotrichum* are also reported as plant pathogens. The consensus ITS2 secondary structure of the pathogenic *C. coccodes* consisted of a conserved core bulge of which radiating three major helixes (H1, H2, and H3), while the ITS2 secondary structure of the endophytic *C. coccodes* as well as for the author's isolate comprised one incomplete (H1) and four complete helixes (H2, H3, H4, and H5) (Fig. 5).

## Methods for studying the chemical diversity of endophytic fungi

Endophytic fungi are known to synthesize a wide range of bioactive molecules with the most diverse applications (Toghueo 2019; Sagita et al. 2021; Zheng et al. 2021). Currently, there are many methods available for evaluation, characterization and isolation of compounds produced by endophytic fungi. Thus, the choice of the best methodology depends on the objectives proposed by the study. To study secondary metabolites, for instance, purified isolates are usually subcultured in liquid medium and incubated for a time course of 7–14 days at 25–28 ºC, under constant agitation (160–180 rpm/min) (Li et al. 2016a, b; An et al. 2020; Tang et al. 2020; Wei et al. 2020). Then, the fermented broth is treated to cause cell lysis, filtered and washed one or more times with different organic solvents, followed by vacuum volatilization or in a water bath to obtain the extracted metabolites, which are separated, identified and tested according to the study (Li et al. 2016a, b; Tang et al. 2020). Moreover, previous screening of fungal endophytes

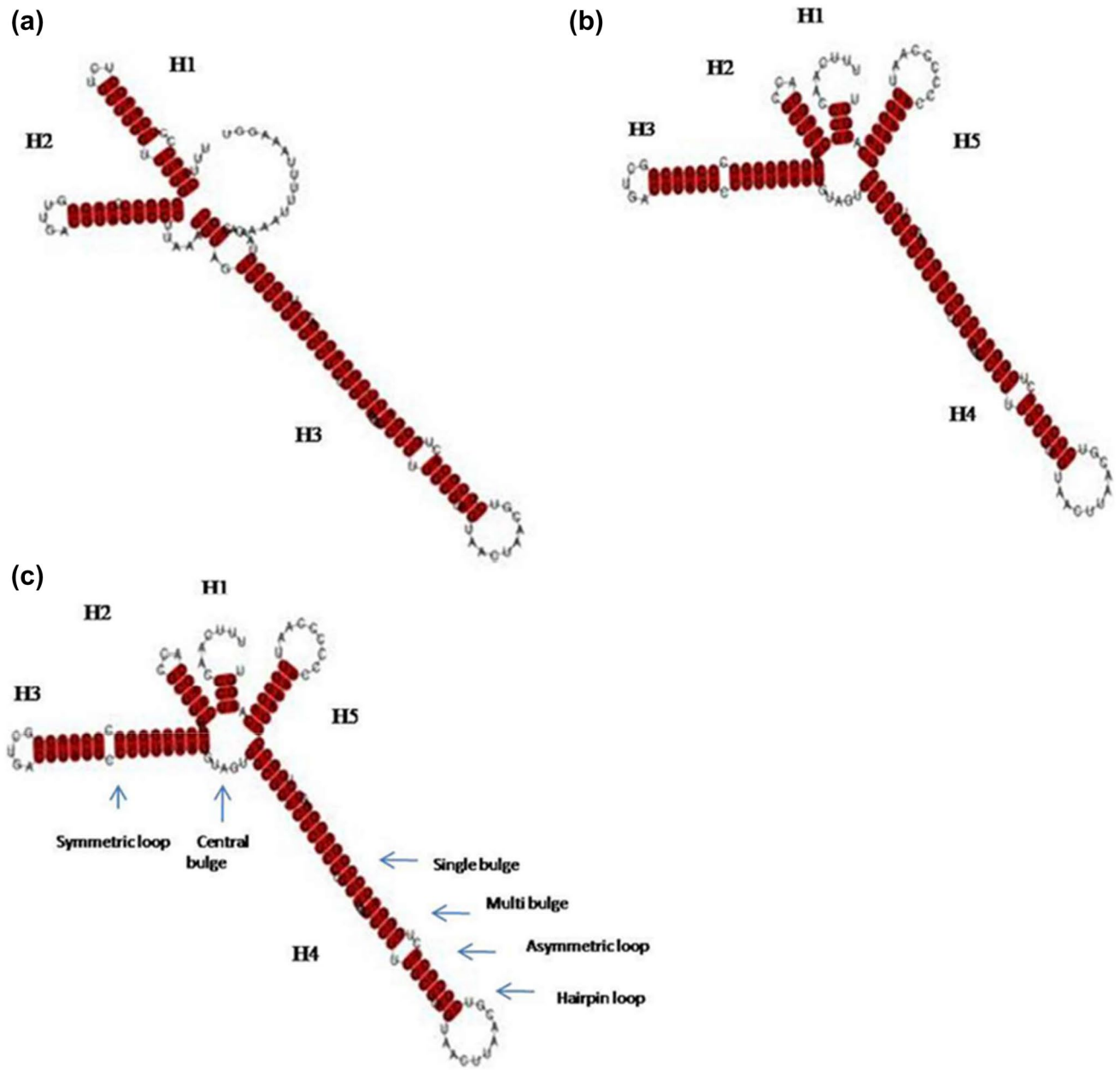


**Fig. 5** ITS2 secondary structures of *C. coccodes* with different life styles showing helixes emerging from a central bulge, **A** pathogenic, **B** endophytic and **C** author's isolate (MN128230.1). Motifs in the structures are indicated by arrow. Retrieved from Talukdar et al. (2021)

for metabolites production are recommended prior to chemical analysis (Luo et al. 2015; Sagita et al. 2021), including PCR detection of specific genes involved in the biosynthesis of metabolites.

Finally, the identification of fungal metabolites can be performed using different approaches. With demystification of omic technologies, metabolomics has been implemented to rapidly expand our understanding of cellular processes. Metabolomics is the large-scale study of end products of all cellular processes, providing a direct functional readout of cellular activity and physiological status at a set time. Thereby, high-performance gas or liquid chromatography (GC and LC, respectively) coupled with mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy (chromatographic profile-guided experiment) are methods largely employed nowadays to analyze bioactive compounds produced by endophytic fungi (Silva et al. 2018; Teimoori-Boghsani et al. 2020; Wei et al. 2020; Sagita et al. 2021).

There is currently little information on endophytic fungi at the omics levels concerning their potential for production of secondary metabolites. *Calcarisporium arbuscula*, an endophytic fungus from the fruit bodies of Russulaceae,

produces a variety of secondary metabolites with anti-cancer, anti-nematode and antibiotic activities (Cheng et al. 2020). The whole genome of *C. arbuscula* NRRL 3705 was sequenced using the PacBio Sequel platform and Illumina NovaSeq PE150, and transcriptomic survey was performed in order to determine its potential for biosynthesis of metabolites. The genome component prediction included the prediction of coding genes, repetitive sequences and non-coding RNAs, and gene clusters for biosynthesis of metabolites by the web-based software antiSMASH database (antibiotics and Secondary Metabolite Analysis 4.0) through the genome mining approach. According to the authors, a comprehensive survey of the genome and transcriptome of this endophytic fungus will allow to understand its capacity to biosynthesize secondary metabolites as well as generate pathways for its exploitation.

## Cultivation-independent methods for the study of endophytic fungi

Recent advances on development of culture-independent methods have allowed the study of microbial communities in a better comprehensive way (Rojas et al. 2019; Ruiz Gómez et al. 2019), since these tools provide a closer detection of the real diversity of microorganisms from environmental samples directly. In addition, these approaches may lead to identification of novel taxa, which are uncultivable through conventional culture-dependent methods and artificial conditions (Forbes et al. 2017).

Cultivation-independent methods have been widely used to study the diversity of fungal communities in a variety of habitats, including soils (Pang et al. 2019), plants (Chi et al. 2019), and hospital environment (Donovan et al. 2018). These methodologies are considered extremely useful to deeply understand the genetic and functional diversity of fungal species (Staniek et al. 2009; Sagita et al. 2021; Chi et al. 2019).

Using culture-dependent in combination with culture-independent (DNA metabarcoding) methods, Chi et al. (2019) accessed the diversity of fungal taxa in *Acanthus ilicifolius*. Through the culture-dependent method, 203 fungal strains were isolated and classified into 30 species, while 111 operational taxonomic units (OTUs) were identified using DNA metabarcoding, which demonstrates the advantages of cultivation-independent methods compared to cultivation-dependent methods in environmental studies of microbial diversity.

Staniek et al. (2009) designed specific primers to study taxol-coding genes in endophytic fungi without the need of cultivation-based procedures mostly associated with methodological and observational biases. Among the culture-independent techniques employed to study endophytic microbial diversity are Denaturing Gradient Gel Electrophoresis—DGGE (Bogas et al. 2015) and DNA metabarcoding (Chi et al. 2018). However, these methodologies are based on PCR amplification of rDNA ITS1-5.8S-ITS2 sequences with specific primers (Chi et al. 2018) that may exclude non-target fungal DNA from environmental samples (O'Donnell 1993). For analysis of PCR products subjected to sequencing and precise species characterization, several pipelines were developed from ITS data, including Plutof, Clotu, PIPITS and CloVR-ITS (Abarenkov et al. 2010; Kumar et al. 2011; White et al. 2013; Gweon et al. 2015). Other bioinformatics pipelines for performing microbiome analysis that includes BioMaS, Mothur and Qiime have also been used to analyze fungal ITS amplicons (Schloss et al. 2009; Caporaso et al. 2010; Fosso et al. 2015) derived from next-generation sequencing (NGS).

Despite advances in culture-independent DNA-based techniques for studying microbial communities, these methods are still under improvement due to methodological limitations. DNA metabarcoding, for instance, usually generates short DNA fragments (~250 bp), making unfeasible the taxa classification at genus and/or species levels (Chi et al. 2019). Anyway, limitations should be considered when choosing an appropriate research methodology.

In the last 2 decades, omics-based approaches and systems biology have emerged, and revolutionary concepts on molecular biology studies are available. Omics involves high-throughput analyses on genomics, transcriptomics, proteomics, metabolomics, lipidomics, and others, which can be integrated through robust systems biology, bioinformatics, and computational tools to study mechanisms of interaction and biological functions of organisms. These approaches are also employed to collective analyses of environmental microbial communities (e.g. metagenomics, metatranscriptomics, and metaproteomics). Thus, NGS (next-generation sequencing) is a massively sequencing technology that has revolutionized the biological sciences and offered ultra-high throughput, scalability, and speed in biological analyses. This technology has been largely used to determine the order of nucleotide sequences in genomes or targeted regions of DNA or RNA, and become an everyday tool to address biological questions.

Zhuoyan et al. (2017) reported the application of high-throughput internal transcribed spacer rRNA metagenomics analysis in deciphering endophytic fungi diversity of *Dendrobium Officinale*. Metagenomic mining of functional genes related to glycyrrhizin synthesis from endophytes of licorice (*Glycyrrhiza uralensis*), a traditional Chinese medicine, was employed by Chen et al. (2021). Glycyrrhizin is the main active ingredient in licorice, a pentacyclic triterpenoid compound with many pharmaceutical functions (e.g. anti-inflammatory, antiviral, and liver protection), which has also been used in the clinical treatment of COVID-19.

**Table 4** Common errors in studies on endophytic fungi and tips to avoid misinterpretation

| Experimental step | Error risks and/or methodological limitations | Preventive measures | References |
|---|---|---|---|
| Identification of plant host(s) and geographic location | Misidentification of the plant species(s) to be analyzed | -Previous knowledge on morphological characteristics of the species(s) to be analyzed<br>-Collect sampling for deposit in herbarium<br>-Document the sampling location and the deposit slip sample<br>-Provide photographic records of the species(s) to be collected, as well as the geographic region | Silva et al. (2004) |
| Collection and processing of plant tissue | Low sampling, sampling of tissues with herbivory or with symptoms of disease may provide erroneous results regarding the diversity of endophytic fungi. Processing time and post-collection storage conditions can also interfere with the diversity of these microorganisms | -Carry out adequate sampling to obtain the greatest possible representation<br>-Collect tissue samples without apparent manifestations of herbivory or disease symptoms<br>-After collection, the plant tissue must be stored in a temperature-controlled container and taken to the laboratory as soon as possible<br>-The surface disinfection process of samples should preferably be carried out within 24 h after collection | Verma et al. (2007) |
| Surface disinfection method | The inappropriate choice of the superficial disinfection protocol may result in non-elimination of the epiphytic mycobiota or loss of the endophytic mycobiota, generating erroneous results | -Conduct previous pilot assays using different superficial disinfection protocols to find the most suitable for the endophytic species and type of tissue analyzed<br>-Carry out at least three successive washes with distilled water<br>-Collect water from the last wash to certify the effectiveness of the surface disinfection method | Saldierna et al. (2020) |
| Plant tissue sampling | Tissue size and sampling of plant numbers may interfere with the observed fungal diversity | -Fragmentation of the fabric to obtain greater coverage<br>-Fragments must be 2–5 mm in diameter<br>-The fabric can be ground or macerated | Gamboa et al. (2002) |
| Culture medium and cultivation | Antibiotic-free culture medium may result in bacterial growth that protrude over the fungal growth. The period and temperature of incubation may also interfere with the fungal diversity under laboratory conditions. Culture-dependent methods are not able to identify fungal taxa not cultivable under laboratory conditions | -Use of antibiotics and colony limiters in the culture medium to prevent bacterial growth<br>-pH adjustment of the culture medium<br>-Choice and control of the appropriate temperature for incubation<br>-Nutritional enrichment/limitation of the culture medium<br>-Use culture medium appropriated to the research objective(s)<br>-Longer incubation time for fungal taxa that demand longer growth time<br>-Use of cultivation-independent methods for a better characterization of the endophytic fungi diversity | Mishra et al. (2012); Sun and Guo (2012); Verma et al. (2014) |

**Table 4** (continued)

| Experimental step | Error risks and/or methodological limitations | Preventive measures | References |
|---|---|---|---|
| Phenotypic identification of isolates | Identification based only on morphological characteristics of isolates is not sufficient to classify the taxa | -Use morphological characteristics prior to sequencing<br>-Use methods for sporulation<br>-Use molecular tools such as Sanger sequencing coupled with or not phenotypic techniques to identification of isolates | Sun and Guo, 2012; Lindahl et al. (2013) |
| Molecular identification of isolates | Contamination of extracted DNA with DNA from other microorganisms, contamination of reagents used and inappropriate choice of primers can result in inaccurate data | -Ensure culture purity<br>-Use negative and positive PCR controls<br>-Choose DNA barcode regions for fungal identification such as 18S rRNA, ITS1, ITS2, 5.8S rRNA and 28S rRNA regions<br>-Choose properly the primers that will be used<br>-Use more than one target gene region to identify the isolates when a single gene region is not enough for taxonomic classification at the species level | Sun and Guo, 2012; Lindahl et al. (2013) |
| Observation of the distribution of endophytic fungi in plant tissue | If the tissue is not treated and stained correctly, there may be misinterpretations | -Select the appropriate clarification method for the species and plant tissue analyzed<br>-Choose properly the colorant to be used<br>-Use fluorescent probes | Detmann et al. (2008) |
| Identification of endophytic fungi from DNA extracted of plant tissue using cultivation-independent methods | -Depending on the technique used, the taxonomic classification may be restricted to higher taxa, such as phylum, class, order, or family<br>-Abundant species may stand out over less abundant ones according to the method implemented, making the characterization of the organisms impossible | -Choose the methodology properly according to the research objectives (e.g. metabarcoding; denaturing gradient gel electrophoresis-DGGE; single sequence repeat<br>-SSR analysis of randomly amplified polymorphic DNA-RAPD, metagenomics)<br>-Combine different methods using a polyphasic approach<br>-Use cultivation-independent and cultivation-dependent methods in association | Sun and Guo, (2012); Verma et al. (2019) |

**Table 4** (continued)

| Experimental step | Error risks and/or methodological limitations | Preventive measures | References |
|---|---|---|---|
| Identification and evaluation of secondary metabolites from cultivable endophytic fungi | Unpurified cultures and inadequate screening processes can lead to contamination by metabolites or enzymes from the host plant and/or other microorganisms, which can generate false-positive results | -Purify the isolated and provide cultivation under controlled conditions<br>-Extract the metabolites to be tested using different organic solvents<br>-Purify the molecules with biological activity to be tested<br>-Include negative control with antifungal agents to detect any transport of secondary metabolites or enzymes from the plant or other contaminating sources that result in the synthesis of secondary metabolites<br>-Provide screening experiments using several specific activity bioassays for each metabolite isolated<br>-Conduct time course experiments to observe if there is an increase in secondary metabolites production compared to an increase in fungal biomass<br>-Include assays under different culture conditions to modulate the production of secondary metabolites (this case should only be considered when there is solid evidence of metabolite production by the isolate)<br>-Use molecular approaches for screening genes responsible for the synthesis of secondary metabolites (e.g. PCR amplification) | Vasundhara et al. (2016); Sagita et al. (2021) |

Illumina-based analysis yields new insights into the diversity and composition of endophytic fungi in cultivated *Huperzia serrata* (Fan et al. 2020). Ascomycota and Basidiomycota were the dominant phyla, and *Cladosporium*, *Oidiodendron*, *Phyllosticta*, *Sebacina* and *Ilyonectria* were the dominant genera according to the taxonomic classification. The heat map relative abundance at the genus level suggested that *H. serrata* had characteristic endophytic fungal microbiomes. In addition, fungal communities were tissue-type and tissue-site specific. According to the authors, this study provided new insights into the complex composition of endophytic fungi in *H. serrata*.

## Final considerations

There are many methodological biases and limitations on endophytic fungi studies from field collection to data processing that jeopardizes information on fungal diversity. In most cases, the cultivation-depend methods are extremely imprecise to access the diversity of endophytic fungi (Taylor et al. 2016; Wu et al. 2019). However, many fungal taxonomists consider the cultivation-independent approaches taxonomically problematic because of the absence of isolated specimens (Lücking et al. 2020). Nevertheless, these techniques cover a high diversity of organisms, including uncultivable fungi by conventional culture-dependent methods and artificial conditions. Therefore, it is strongly recommended a polyphasic approach combining different methodologies to produce meaningful information on endophytic fungi. Finally, tips and possibilities to minimize some limitations on endophytic fungi studies are summarized in Table 4 as a contribution to improve future studies.

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**Author contributions** JBAdR: conceptualization, writing–original draft, writing–review and editing. ASL: writing–review and editing. HMMdV: conceptualization, writing–review and editing.

## Declarations

**Conflict of interest** The authors declare that the work was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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