

A review: Molecular identification of orchid mycorrhiza

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Abstract: Orchids are a diverse and widespread family of flowering plants, with over 25,000 known species and more than 100,000 hybrids and cultivars. Orchids are characterised by their often showy and highly specialised flowers and have unique and intricate floral. Orchids are known to be highly dependent on their mycorrhizal fungi for nutrient uptake, especially during the early stages of their development. Orchid seeds lack the endosperm present in most other seeds, which means they cannot germinate without a source of nutrition. The relationship between orchids and mycorrhiza is known as orchid mycorrhizae or orchid mycorrhiza. In orchid mycorrhiza, the orchid plant forms a mutualistic relationship with certain species of fungi that are able to penetrate the orchid's roots and colonise its tissues to provide the orchid with essential nutrients. Orchid mycorrhizal fungi are often highly specific, meaning that they can only form partnerships with certain orchid species, and vice versa. The importance of mycorrhizal fungi in the orchid life cycle is crucial from both evolutionary and ecological standpoints. Therefore, it is essential to acquire a thorough comprehension of this relationship and develop methodologies for isolating, identifying, and preserving significant fungal strains that are associated with different orchid species. In recent years, there has been a considerable increase in research concentration on mycorrhizal interactions in orchids. However, certain inquiries remain unresolved pertaining to the fungal communities associated with orchids as well as the divergences noticed across different species and geographical locales. The present paper provides a thorough, and extensive analysis of the fungal life associated with orchids. This article presents a succinct overview of the molecular techniques utilised by researchers globally to isolate and identify peloton-forming fungi in both temperate terrestrial and tropical orchids. The review begins by providing a concise introduction to the background material regarding the wide range of fungal species that are linked with orchids. It then proceeds to explore the topic of orchid mycorrhizal fungi (OMF) and orchid non-mycorrhizal fungi (ONF). The subsequent analysis explores the crucial function that orchid mycorrhizal fungi play in the processes of seed germination and development. Moreover, the study elaborates on the methodologies utilised for isolating fungi, extracting fungal DNA, selecting primers, amplifying DNA and subsequent analysis sequence data. This article considers several molecular identification approaches that are used in studying orchid endophytic mycorrhizal. Using molecular approaches, orchid mycorrhizal can be further explored and identified.

1. Introduction

The Orchidaceae family is considered the second largest among flowering plants, with its size exceeded only by the Asteraceae family (Givnish *et al.*, 2015). According to Govaerts *et al.* (2017), the number of recognised orchid species is estimated to be 29,199. The decision to classify all orchids under Appendices I and II by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in 2017 effectively prohibited the illicit trade of these plants (Hinsley *et al.*, 2018). Based on the evaluations made on a total of 1770 species of orchids, it has been determined that approximately 46.5% of these species are classified under the categories of vulnerability, endangered, or critical endangered as reported by the International Union for Conservation of Nature (IUCN, 2021). This precarious state remains due to a variety of variables, including their difficult germination process and human intervention, such as overcollection caused by economic and horticultural needs (Pujasatria *et al.*, 2020; Suresh *et al.*, 2023).

Orchids are extraordinarily important for biodiversity, conservation, and producing a vast array of therapeutic substances, nutritious foods, and ornamental plants (Hinsley *et al.*, 2018). Orchid conservationists strive to manage market needs and biodiversity on a global scale, which would need large-scale production (Pujasatria *et al.*, 2020). Numerous species encounter the peril of extinction; however, orchids adopt two distinct evolutionary strategies, namely sympodial growth and monopodial development, which are regulated by a diverse array of endophytic fungus species. These techniques serve to extend the longevity of orchids as herbaceous plants. (Srivastava, 2018). Orchid endophyte has a different way of penetrating and colonising their host, which makes them different from another fungal pathogen. For example, orchid fungi endophytes enter through stomata laterally in the anticlinal epidermal cell. They remain intracellularly in the shoot without colonising the cell. In contrast, pathogen fungi enter directly from the cell wall and typically grow extracellularly, potentially causing harm to the host (Sarsaiya *et al.*, 2019).

Diverse fungal taxa include mutualistic mycorrhiza, endophytic fungi and considerably diverse as well as non-mycorrhizal fungal associates. The role of the root-allied fungi is not well understood. According to

Lee and Yeung (2018), some of these fungi may supply organic carbon, nutrients, and water to the orchid, but the degree of this transfer is typically unknown. Numerous report on specific mycorrhizal fungi also shows the ability to stimulate the embryo's development and supply it with necessary nutrients, allowing the orchid seeds to germinate (Liu *et al.*, 2010; Zhang *et al.*, 2016; Shao *et al.*, 2017; Herrera *et al.*, 2019; Shah *et al.*, 2019; Suresh *et al.*, 2023).

In recent years, there has been a significant transformation in the application of molecular techniques. The identification of fungi within roots has been accomplished through the application of polymerase chain reaction (PCR) techniques, employing fungal-specific primers (Gardes and Bruns, 1993). Such methods have been used to characterize mycobionts of Orchidaceae, Taylor and Bruns (1999) employed these techniques to characterise mycobionts of Orchidaceae, thereby removing the laborious process of culturing. The region that is most frequently studied is the nuclear ribosomal internal transcriber spaces (ITS). Therefore, there is a want for molecular techniques capable of discerning distinct fungal species in cases where numerous fungal species are present in a single plant.

The present review has provided an overview of the principal discoveries and methodologies utilised in the discipline, underscoring the significance of molecular techniques such as fungal DNA extraction, primer selection, polymerase chain reaction (PCR), and high throughput sequencing (HTS) in discerning the taxonomy of mycorrhizal fungi and elucidating the underlying molecular mechanisms that regulate these symbiotic relationships. Furthermore, the utilisation of molecular method has provided researchers with enhanced capabilities to explore the extensive range of orchid mycorrhizal variety. The investigation has not only shown evolutionary relationship but has also yielded significant insights into ecological and conservation concerns. A thorough comprehension of mycorrhizal connections is essential for the efficient preservation of orchid species. In addition, the exploration of orchid mycorrhizal fungus in the fields of biotechnology and agriculture has resulted in the identification of new and important mycorrhizal fungi. In the context of identifying orchid mycorrhizal fungi, many methodologies are routinely applied, encompassing the isolation and cultivation of fungi, microscopic analysis and molecular studies. The ongoing refining

and improvement of these techniques play a crucial role in further our understanding and fascinating associations between orchids and their mycorrhizal fungus.

2. Orchid and its fungi diversity

Orchids form a unique symbiotic relationship with the plant and animal species present in forest habitats in order to acquire nutrients, facilitate their own development, and facilitate the process of pollination. The mycorrhizal fungi, which exhibit symbiotic germination, are of significant importance in facilitating embryo development and supply vital nutrients within the natural environment. This symbiotic relationship is crucial in the effective germination of orchid seeds (Liu *et al.*, 2010; Herrera *et al.*, 2019). Fungi have a crucial role as the principal provider of essential nutrients for developing Orchidaceae plants, especially in setting characterised by low nutrients availability (Long *et al.*, 2022).

Orchids interact with a smaller number of mycorrhizal fungi than other mycorrhizal plants, with greater specificity for orchid mycorrhizal fungi than ectomycorrhizae, arbuscular mycorrhizae, and even ericoid mycorrhizal fungi. In symbiotic connection, fungi provide plants with water and mineral nutrients (especially phosphorus) while protecting them from biotic and abiotic stresses. In exchange, plant hosts provide carbon from photosynthesis to the fungi (Rasmussen, 1995; Tedersoo *et al.*, 2017).

Many orchid species cannot commence germination or grow without their compatible symbiotic fungus (Rasmussen, 1995; Davis *et al.*, 2015; Fay, 2018; Attri, 2022), as their specificity of mycorrhizal connections that permit *in situ* symbiotic seed germination in orchids is frequently so rigorous. The aforementioned circumstance has stimulated inquiries into the importance of fungus in symbiotic relationships that are equally crucial and beneficial for the *ex-situ* preservation of orchids, specifically in the context of reintroduction endeavours. The first recorded evidence of a mycorrhizal fungus in an orchid may be traced back to the year 1824, as documented by the renowned German naturalist Heinrich Link. Nevertheless, the specific function of the fungus remained ambiguous until the early 1900s when Noël Bernard established a scientific correlation between filamentous fungi and the process of seed

germination (Arditti and Pridgeon, 1997). Following this, in the early 1900s, the study of orchid endophytes emerged as a significant area of interest within the field of orchid biology research. Chand *et al.* (2020) conducted a comprehensive investigation wherein they isolated and identified many orchid endophytes, and thoroughly evaluated their probable role in orchid symbiosis.

Orchids frequently establish symbiotic relationships with fungus that display substantial evolutionary and ecological variability. Epiphytic orchids exhibit a prevalence of both Basidiomycota and Ascomycota in their aerial roots as well as subterranean roots or rhizomes, while Chytridiomycota, Glomeromycota, Zygomycota, or Mucoromycota are present in comparatively smaller quantities (Waud *et al.*, 2014; Cevallos *et al.*, 2017; Egidi *et al.*, 2018; Novotná *et al.*, 2018). The classification of orchid fungus is determined by the existence or absence of functional pelotons within cortical cells, leading to the categorization of orchid mycorrhizal fungi (OMF) or orchid non-mycorrhizal fungi (ONF) (Li *et al.*, 2021).

3. Orchid mycorrhizal fungi (OMF) and Orchid non-mycorrhizal fungi (ONF)

The phenomenon referred to as “orchid mycorrhiza” pertains to the symbiotic relationship established between the orchid plant and many fungal species that are capable of cohabiting within its root system. The germination of an orchid mycorrhizal fungus (OMF), and these seeds rely on one or more OMF’s for sustenance during their whole life (Bidartondo and Read, 2008). Within the realm of fungi, a subset of these organisms can be classified as transient, denoting their inability to maintain a sustained presence within the developing and maturing tissues of orchids. Conversely, there exist other fungi that establish more long-lasting associations with these plants. According to Lee and Yeung (2018), during the maturation process of orchids, specific fungi that play a role in facilitating germination persist as “permanent residents” whereas other fungi initiate germination and are subsequently replaced by different fungal partners.

The identification of coiled pelotons within cortical root cells is recognised as a characteristic feature of orchid mycorrhizal fungus (OMF), as examined in research undertaken by Dearnaley *et al.* (2016) as well as Rasmussen (1995). In contrast,

orchid mycorrhizal fungi (ONF) pertain to a distinct classification of endophytic fungi that reside within the roots or other tissues of orchids at specified phases of their life cycle. Nevertheless, it is imperative to elucidate that oligotrophic nitrogen-fixation bacteria (ONFs) are devoid of peloton-like structures and do not elicit any noticeable pathogenic consequences in the host plants. The aforementioned phenomenon has been emphasised in scientific inquiries conducted by Sisti *et al.* (2019) and Selosse *et al.* (2018).

Several investigations, like those conducted by Herrera *et al.* (2019) and Waterman *et al.* (2011), have shown empirical evidence indicating the involvement of certain orchid mycorrhizal fungi (OMFs) in the process of decomposition. The observed mycorrhizal fungi (OMFs) have been documented to facilitate the decomposition of nearby substrates and provide essential nutrients to orchids. It is important to acknowledge that specific obligatory mycoheterotrophic fungi (OMFs) may have experienced evolutionary shifts from ancestral obligate non-photosynthetic fungus (ONFs), gradually developing mycorrhizal capacities. The aforementioned phenomenon has been thoroughly investigated in scholarly studies conducted by Selosse *et al.* (2018) and Wang *et al.* (2021).

The classification of orchid mycorrhizal fungus (OMF) has a wide range of fungal species, consisting of at least 17 families from the basidiomycetes group and five families or genera from the ascomycetes group, as documented by Dearnaley (2007) and Dearnaley *et al.* (2012). Within this set, there are several noteworthy groups, namely Ceratobasidiaceae (Cantharellales), Tulasnellaceae, and Serendipitaceae, which were previously referred to as the Sebaciniales clade B. The classification of these groupings as Rhizoctonia-type Basidiomycetes is largely acknowledged in the scientific community, as evidenced by multiple research (Rasmussen, 1995; Bayman and Otero, 2006; Sisti *et al.*, 2019; Selosse *et al.*, 2018; Jędryczka *et al.*, 2023). Basidiomycetes and Ascomycetes, which are widely distributed in terrestrial ecosystems and cultivated plants globally, have notable associations with orchids (Trivedi *et al.*, 2020; Wang *et al.*, 2019).

The significance of orchid mycorrhizal fungi (OMF) in specific microenvironments cannot be understated, as they play a crucial role in promoting the germination of orchid seeds and the subsequent growth of orchid seedlings. As a result, geographical

areas that display a significant occurrence of orchid mycorrhizal fungi (OMF) tend to showcase a higher range of orchid species, as documented by Li *et al.* (2021). In their study, Hemrová *et al.* (2019) conducted germination tests and developed species distribution models that integrated multiple habitat parameters. The results of their study emphasized the crucial significance of fungal symbionts in influencing the spatial distribution of orchids on a large geographical scale. Furthermore, McCormick *et al.* (2019) and other scientific inquiries have provided substantial data supporting a strong and positive association between the prevalence of mycoheterotrophic orchids, which depend on fungi for nourishment, and the existence of OMF. The cumulative evidence suggests that OMF has a significant role in shaping the population dynamics of orchids.

4. Orchid mycorrhizal and its roles in seed germination and development

In general, asymbiotic or symbiotic procedures can be used to germinate orchid seeds (Yam and Arditti, 2009). It has been demonstrated that asymbiotic seed germination is an effective method for producing plantlets of numerous orchid species for both commercial and conservation.

It was believed that root orchid mycorrhizal fungi are the actual source of seed-germinating orchid mycorrhizal fungi (Rasmussen, 1995). Root fungal endophytes are seen as advantageous plant residents that may increase their productivity and eventually support ecological functions. Roots of mature plants have provided fungi that have been isolated and tested, with several successes have been attained employing these fungi (Nontachaiyapoom *et al.*, 2011; Sebastián *et al.*, 2014). The *in situ/ex-situ* seed baiting technique has been increasingly popular in recent years as a means of obtaining efficacious fungi that facilitate seed germination. According to previous studies conducted by Zhou and Gao (2016) and Rasmussen and Whigham (1993), it has been observed that fungus obtained from naturally occurring protocorms or seedlings possess the capacity to induce seed germination and facilitate the subsequent development of seedlings (Table 1). Shao *et al.* (2020) conducted a conservation project with the objective of protecting *Dendrobium* species

Table 1 a - List of orchid mycorrhizal fungi that had been identified and their roles in orchid micropropagation

Orchid	Fungi sp.	Roles	References
<i>Vanda wightii</i> (E)	<i>Ceratobasidium</i> sp.	Seed germination	Suresh et al. (2023)
<i>Paphiopedilum barbigerum</i> (T)	<i>Epulorhiza</i> sp.	Seed germination and seedling development	Tian et al. (2022)
<i>Serapias vomeracea</i> (T)	<i>Tulasnella calospora</i>	Seed germination and seedling development	Ghirardo et al. (2020)
<i>Epidendrum secundum</i> (T)	<i>Ceratobasidium</i> sp., <i>Sebacina vermifera</i>	Seed germination and seedling development	Durán-López et al. (2019)
<i>Dactylorhiza majalis</i> (T)	<i>Piriformospora indica</i>	Seed germination	Shah et al. (2019)
<i>Chloraea gaviu</i> (T)	<i>Tulasnella</i> sp.	Seed germination	Herrera et al. (2017)
<i>Aerides multiflora</i> (E)	<i>Ceratobasidium</i> sp.	Seed germination	Bhatti et al. (2017)
<i>Dendrobium friedericksianum</i> (E)	<i>Tulasnella</i> sp., <i>Tulasnellaceae Rigidoporus vinctus</i> , <i>Polyporales Ceratobasidium</i> sp., <i>Tulasnellaceae Flavodon flavus</i> , <i>Polyporales Nigroporus vinosus</i> , <i>Polyporales Corioloopsis retropicta</i> , <i>Polyporales Valsa eugeniae</i> , <i>Diaporthales</i> .	Seed germination and seedling development	Agustini et al. (2016)
<i>Paphiopedilum villosum</i> (E)	<i>Tulasnella</i> sp., <i>Tulasnellaceae</i>	Seed germination	Khamchatra et al. (2016)
<i>Dendrobium lancifolium</i> (E)	<i>Rhizoctonia</i> sp.	Seed germination	Agustini et al. (2016)
<i>Liparis japonica</i> (T)	<i>Rhizoctonia</i> sp.	Seed germination	Ding et al. (2014)
<i>Dendrobium aphyllum</i> (E)	<i>Tulasnella</i> sp., <i>Trichoderma</i> sp.	Seed germination	Zi et al. (2014)
<i>Dendrobium aphyllum</i> (E), <i>Dendrobium devianum</i> (E), and <i>Cymbidium manni</i> (E)	<i>Tulasnella</i> sp., <i>Epulorhiza</i> sp.	Seedling growth	Zi et al. (2014)
<i>Dendrobium officinal</i> (E)	<i>Tulasnella</i> sp.	Seed germination and seedling growth	Ming et al. (2014)
<i>Dendrobium nobile</i> (E), <i>Dendrobium</i> <i>Chrysotoxum</i> (E), <i>Dendrobium</i> <i>falconer</i> (E), <i>Dendrobium</i> <i>aphyllum</i> (E)	<i>Xylariaceae</i> sp.	Seed germination	Chen et al. (2013)
<i>Dendrobium crumenatum</i> (E)	<i>Guignardia endophyllicola</i>	Seed germination	Mangunwardoyo et al. (2011)
<i>Pecteilis susannae</i> (L.)	<i>Epulorhiza</i> sp.	Seed germination and development	Chutima et al. (2011)

E= epiphytes; T= Terrestrial.

Table 1 b - List of orchid mycorrhizal fungi that had been identified and their roles in orchid micropropagation

Orchid	Fungi sp.	Roles	References
<i>Dendrobium nobile</i> (E)	<i>Leptodontidium</i>	Seedling development	Hou and Guo (2009)
<i>Cymbidium eburneum</i> (E)	<i>Alternaria</i> sp., <i>Chaetomium</i> sp, <i>Fusarium</i> sp.	Vegetative growth	Zhao and Liu (2008)
<i>Gastrodia elata</i> (T)	<i>Mycena osmundicola</i>	Seed germination	Kim <i>et al.</i> (2006)
<i>Cymbidium goeringii</i> (T)	<i>Rhizoctonia</i> sp	Seedling development	Jianrong <i>et al.</i> (2005)
<i>Gastrodia elata</i> (T)	<i>Mycena osmundicolor</i>	Seed germination	Hong <i>et al.</i> (2002)
<i>Paphiopedilum armeniacum</i> (T)	<i>Phacodium</i> sp.	Seedling development	Ming and Zhou (2001)
<i>Cypripedium reginae</i> (T)	<i>Fusarium</i> sp.	Seed germination	Warcup (1981)
<i>Dendrobium discolor</i> (E),	<i>Tulasnella cruciate</i> , <i>Tulasnella irregularis</i> , <i>Tulasnella allantospora</i>	Seed germination	Warcup (1981)
<i>Calochilus</i> sp. (T), <i>Diuris maculata</i> Sm. (T), <i>Spiranthes sinensis</i> (T)	<i>Tulasnella asymmetrica</i> , <i>Tulasnella cruciate</i> , <i>Tulasnella irregularis</i> , <i>Tulasnella violea</i> , <i>Tulasnella allantospora</i>	Seed germination	Warcup (1981)
<i>Diuris sulphurea</i> . R.Br. (T)	<i>Tulasnella asymmetrica</i>	Seed germination	Warcup (1981)
<i>Orthocersa strictum</i> (T)	<i>Tulasnella asymmetrica</i> , <i>Tulasnella cruciate</i> , <i>Tulasnella irregularis</i> , <i>Tulasnella violea</i>	Seed germination	Warcup (1981)
<i>Thelymitra ixoides</i> (T)	<i>Tulasnella asymmetrica</i> , <i>Tulasnella cruciata</i>	Seed germination	Warcup (1981)
<i>Thelymitra flexuosa</i> (T)	<i>Tulasnella irregularis</i> , <i>Tulasnella cruciata</i>	Seed germination	Warcup (1981)
<i>Thelymitra media</i> (T)	<i>Tulasnella violea</i> , <i>Tulasnella asymmetrica</i>	Seed germination	Warcup (1981)
<i>Thelymitra carnea</i> (T)	<i>Tulasnella allantospora</i> , <i>Tulasnella violea</i>	Seed germination	Warcup (1981)

E= epiphytes; T= Terrestrial.

that have been excessively harvested. They effectively isolated and obtained fungi that enhance germination for several *Dendrobium* species using the seed baiting approach, as described by Huang *et al.* (2018).

5. Fungal DNA extraction

There are various methodologies commonly employed for the isolation of orchid mycorrhizal fungus from orchid plants. These methodologies encompass the isolation of complete tissue or tissue segments, *in situ* seedlings, trapping isolation, and isolation from a solitary peloton. Among these methods, the technique of isolating a single peloton, which involves micromanipulation-based isolation from host cells, is widely regarded as the most reliable and precise approach for extracting endophytic mycorrhizal fungi (Zettler *et al.*, 2003; Batty *et al.*, 2006; Zi *et al.*, 2014; Zettler and Corey, 2018). The prevailing conventional method for molecular identification of orchid mycorrhizal fungus generally entails the extraction of DNA from agar plates or liquid cultures, as opposed to direct extraction from orchid roots (Zettler and Corey, 2018).

The fungal cell wall primarily consists of around 80-90% polysaccharides, inorganic ions, lipids, polyphosphates, and proteins, which together form the matrix that binds the wall. This type of cell wall also can be characterized by microfibrillar components like chitin, β -glucan, and/or cellulose, which pose challenges in DNA extraction (Turzhanova *et al.*, 2018). Moreover, the presence of a substantial quantity of secondary metabolites, such as melanin, can impede subsequent reactions (Fernandez *et al.*, 2016; Janowski *et al.*, 2019). This becomes a major challenge in DNA extraction of fungi as it has a robust cell walls that are resistant to lysis method (Jiang *et al.*, 2011). The isolating nucleic acids from fungi, often necessitates the incorporation of additional lysis steps, which can include enzymatic lysis, mechanical homogenization, sonication, or the use of potentially harmful chemicals (Turzhanova *et al.*, 2018).

DNA samples were gathered over a period of 15 years, during which a diverse range of extraction procedures were utilised to extract fungal DNA. Nevertheless, the extraction of DNA from the various types of fungi encountered does not have a universally optimised approach. The standard

procedure for the extraction of fungal DNA typically encompasses several sequential stages. These stages involve the cultivation of fungi in either liquid or solid growth media, disruption of the fungal cell wall, elimination of proteins using phenol and chloroform, and subsequent isolation of DNA through precipitation with ethanol or isopropanol (Faggi *et al.*, 2005). Even though the presence of polysaccharide and polyphenolic compound in the fungi may inhibit the activity and effect of DNA polymerase, but they can be easily removed by either using a vacuum or spin column and by mixing the sample with bovine serum albumin (BSA), β -mercaptoethanol (β ME), N-trimethyl ammonium bromide (CTAB) and Polyvinylpyrrolidone (PVP) (Tripathy *et al.*, 2017).

A variety of methodologies have been devised to isolate DNA from fungal tissues, and the most efficacious DNA extraction procedures frequently integrate physical methodologies (such as microwave treatment, freeze/thaw cycles, homogenization using glass beads, and grinding in liquid nitrogen) with enzymatic approaches (including gluconases, chitinases, and proteases) (Zhang *et al.*, 2010). The exists variety of ways for extracting DNA and among them, the CTAB approach (Gardes and Bruns, 1993) is frequently utilised.

Additional alternatives for fungal genomic DNA isolation kits are the Omega Fungal E.Z.N.A kit (manufactured by Omega Biotech, Doraville, GA, USA), the Qiagen Plant DNeasy kit, Genomic Tip kits (Qiagen, Valencia, Cam USA), or Sangin Biotech Rapid Fungi Genomic DNA isolation kits (Long *et al.*, 2022). In order to ascertain the effectiveness of a DNA extraction technique, it is important to evaluate both the quality and quantity of the DNA obtained. The concentration of DNA in the samples was assessed by employing spectrophotometry at wavelength of 260 nm, with measurement expressed in units of nanograms per microliter (ng/ μ L). In addition, the assessment of DNA purity was conducted by determining the A260/A280 ratio and A260/280 ratio utilising either a UV-VIS spectrophotometer or Nanodrop device (Thermo Electron Scientific Instruments LLC, USA). Generally, the A260/A280 ratio exceeded 1.8 suggesting that the DNA was largely devoid of proteins. In terms of the A260/A230 ratio, if it was approximately 2, that indicate the samples did not contain significant impurities such as carbohydrates, peptides, phenols, salts, or aromatic compounds (Turzhanova *et al.*, 2018). Furthermore, the quality of the DNA also can be evaluated through

electrophoresis after PCR amplification of the genomic DNA, using gene-specific primers (Tripathy *et al.*, 2017).

The standard CTAB phenol-chloroform extraction procedure has proven effective across a wide range of species (Strugnell *et al.*, 2006; Reineke *et al.*, 1998) and produce a high purity of DNA (Zettler and Corey 2018). Study by Turzhanova *et al.* (2018) on optimization of DNA extraction methods of fungi has shown that CTAB-method and DNeasy Plant mini Kit (Qiagen) resulted a highest DNA quality, while SDS method resulted in the lowest sample yields and quality. However, CTAB-method uses toxic chemicals and requires a significant amount of bench time, both limiting its applicability when scaling up for big comparative research (Schiebelhut *et al.*, 2017). Nowadays, commercial DNA extraction kits are more desirable since they reduce exposure to toxic

chemicals and allow for faster extraction periods. These kits could offer a range of low- to high-throughput processing, vary in price from quite inexpensive to highly costly, and may require some specialist gear. Table 2 below shows a list of extraction methods and kits used in the extraction method of DNA orchid fungi.

A large percentage of orchid mycorrhizal fungi are mycelia sterilia. Conventional techniques have led to a paraphyletic taxonomy in which unrelated fungi are grouped together, requiring molecular techniques for accurate identification, phylogenetic inference, and genetic relatedness (Sen *et al.*, 1999; Otero *et al.*, 2002; Shan *et al.*, 2002; Yagame *et al.*, 2008). Molecular sequencing, microscopic examination, and biochemical analysis were among the most used methods to identify mycorrhizal fungi. For fungi identification by morphological characterisation, it

Table 2 - Types of manual DNA extraction protocol used to extract mycorrhizal fungi DNA

Protocol name	Abbreviation	Chemistry/mechanism	Kits/supplies required	DNA extraction time	References
Cetytrimethyl ammonium bromide (CTAB)-Phenol-chloroform	CTAB	CTAB lysis, followed by phenol chloroform purification step	All reagents are made in-house	1 hour 30 min	Sambrook <i>et al.</i> (2001); Dawson <i>et al.</i> (1998)
Sodium Dodecyl Sulfate	SDS lysis, followed by phenol chloroform purification step	SDS and mercaptoethanol lysis, followed by chloroform purification step	All reagents are made in-house	1 hour 5 min	Turzhanova <i>et al.</i> (2018)
Phenol Chloroform Isoamyl alcohol extraction method	PCI	Buffer lysis. Followed by Phenol/chloroform /isoamyl alcohol purification step	All reagents are made in-house	2 hour 10 min	Varma and Kwon Chung (1991)
EZNA SP Fungal DNA	Omega Fungal EZNA	Silica based purification system	Omega Fungal EZNA kit (Omega Biotek, Doraville, GA, USA)	45 min	Omega (2019)
Qiaamp Mini Kit (Qiagen)	QIAamp Mini kit	Silica based purification system	Qiaamp Mini Kit (Qiagen)	35 min	Turzhanova <i>et al.</i> (2018)
Fungi/Yeast Genomic DNA Isolation (Norgen)*	Fungi/Yeast Genomic DNA Isolation	Silica based purification system	Fungi/Yeast Genomic DNA Isolation Kit (Norgen)	More than 2 hours	Kumar and Mugunthan (2018)

* Modified method.

can be conducted alone or in combination with molecular analysis, and usually, most research will use both combination methods in identifying mycorrhizal fungi. However, the orchid fungus is notoriously difficult to be determined at the species level because they do not sporulate readily on cultures (Boddington and Dearnaley, 2008; Ko *et al.*, 2011; Ma *et al.*, 2015).

6. Primer selection for fungal amplification

After the completion of DNA extraction from orchid mycorrhizal fungi, the subsequent step involves the amplification of fungal DNA through the utilisation of a polymerase chain reaction (PCR) technique. This amplification process necessitates the use of primers that are specifically designed to target the ribosomal DNA (rDNA) region. The rDNA cluster consists of several components, including 18S rDNA, 5.8S rDNA, 28S rDNA, the External Transcribed Spacer (ETS), and Internal Transcribed Spacer 1 and 2, which are generally referred to as ITS1 and ITS2. The utilisation of the ITS region for molecular identification of fungi can be traced back to the early 1990s, as shown by Horton and Bruns (2001) and Seifert (2009).

The utilisation of the ITS region for molecular identification is of great significance in fungal identification, principally owing to the inclusion of two remarkably variable spacers, namely ITS1 and ITS2, which frequently exhibit species-specific characteristics either independently or in conjunction. Moreover, it includes the 5.8S gene, which is renowned for its exceptional level of conservation. The high degree of sequence conservation observed in the adjacent genes, along with their designation as the region undergoing the most rapid evolution and the existence of multiple copies of the ribosomal operon, facilitates the efficiency of primer design and PCR amplification for the ITS region (Bengtsson Palme *et al.*, 2013; Fajarningsih, 2016; Raja *et al.*, 2017). These two spacers are copied from the ribosomal DNA, and when the ribosomal RNAs complete, they are removed from the rRNAs. Since the spacers are not used in the final structure of the ribosome, they are not strongly selected against mutations. Therefore, the identification of mycorrhizal fungus is considered efficient by using a region-specific to eukaryotes (Tedersoo and Nilsson, 2016).

The nuclear ribosomal RNA genes, including the small subunit (SSU) (18S) and large subunit (LSU) (28S) are commonly utilised in scientific investigations pertaining to aquatic fungus and arbuscular mycorrhizal fungi. Nevertheless, in the case of ascomycetes and basidiomycetes, these markers generally offer taxonomic insights primarily at level beyond the species, and occasionally at the genus level. This problem is caused by the fact that the SSU and LSU sequences of the many species that belong to these fungal groupings have only minute to nonexistent differences between them. Because of this, precise distinction becomes a challenging obstacle. According to the findings of the research carried out by Nilsson *et al.* (2019), the ability of SSU, LSU, and protein-coding genes like the RNA polymerase gene RPB2 to be aligned across different fungal phyla is a significant benefit offered by these types of genes. This makes it possible to analyse large-scale phylogenetic relationships at the phylum and order levels, which is something that the ITS region normally has difficulty accomplishing without very identical reference sequences (Větrovský *et al.*, 2016). Because the ITS region often ranges in length from 500-700 bases, the majority of high-throughput sequencing (HTS) studies concentrate on the shorter ITS1 or ITS2 subregions, which typically range in length from 250-400 bases. This constraint is the result of the fact that the ITS region is normally between 500-700 bases in length. According to Tedersoo *et al.* (2015), the ITS2 subregion in particular stands out due to the fact that it exhibits lesser length fluctuations and more universal primer sites. This, in turn, results in reduced taxonomic bias.

The ITS1 and ITS2 subregions have demonstrated their suitability for second generation High-Throughput Sequencing (HTS) techniques. However, third generation methodologies, such as those utilising PacBiosciences (PacBio) and Oxford Nanopore platforms, provide the ability to target the complete ITS region, as well as segments or even the entire adjacent rRNA genes (Nilsson *et al.*, 2019). Targeting the entire Internal Transcribed Spacer (ITS) area, rather than its subregions has several advantages, including improved taxonomic accuracy and less amplification of non-viable organism. Nevertheless, one limitation of this methodology is its reduced efficacy when utilised on materials of subpar quality, such as ancient herbarium specimens, which degrade to a degree where doing ITS DNA sequencing becomes impractical (Tedersoo *et al.*,

2017). According to study conducted by Nilsson *et al.* (2019), it is recommended to allocate a significant amount of effort to the analysis and selection of primers, this is due to the fact that only a limited number of primers have the capability to amplify over 90% of fungal groups. Additionally, the process of primer selection necessitates meticulous examination of the target taxa, as highlighted by Tedersoo *et al.* (2015). The following table 3 and 4 show an illustrative depiction of ITS primers together with their corresponding sequences.

7. Identified fungal from orchid root by using internal transcribe region

Gardes and Bruns (1993) and White *et al.* (1990) have produced well recognised primers in the field of fungal ecology for species-level identification based on sequencing. These primers, namely ITS1, ITS2, ITS3, ITS4, ITS1F, ITS86F, and cNL2f, are considered to be broad-spectrum primers. The ITS1 and ITS4 primers are commonly employed as standard primers in numerous laboratories (Fajarningsih, 2016). The list of endophytic fungi that have been isolated and

identified from orchid roots is presented in table 5. This was accomplished by employing a broad-spectrum primer (ITS1 and ITS4).

However, some primers are designed to be specific. For example, the ITS86F primers are used primarily for medically important fungal pathogen, but they are rarely used in mycorrhizal identification, especially fungi communities from environmental samples. In orchid mycorrhizal fungi identification, the ITS1-F is one of the most effective primers for the ITS region amplification, especially for Eumycota. For example, the primer ITS1-F and ITS4 always used in pair to identified an *Fusarium* sp. as in the study by Sukarno *et al.* (2023), where they manage to identified several species of *Fusarium* using this primer combination. The ITS1-F and ITS4-B primer were designed to be specific basidiomycetes (Gardes and Bruns, 1993). Besides that, both primers can minimise plant sequence amplification (Taylor and McCormick, 2008). However, this primer is ineffective in amplifying some species of Tulasnellaceae that belong to Basidiomycota phylum, as their nuclear ribosomal is evolving rapidly and some primers are typically conserved along the Eumycota are not maintained in Tulasnellaceae

Table 3 - List of recommended primer for identification of orchid mycorrhizal

Primer	Sequence (5'-3')	References
Modified ITS1ngs	TCCGTAGGTGAACCTGC	Oja <i>et al.</i> (2014)
Modified ITS1Fngs	GGTCATTTAGAGGAAGTAA	Oja <i>et al.</i> (2014)
Modified ITS4ngs	TCCTSCGCTTATTGATATGC	Oja <i>et al.</i> (2014)
ITS4Tul2	TTCTTTTCTCCGCTGAWTA	Oja <i>et al.</i> (2014)
TW14ngs	CTATCCTGRGRGAAAYTTC	Tedersoo <i>et al.</i> (2014)
fITS9	GAACGCAGCRAAIIGYGA	Ihrmark <i>et al.</i> (2012)
gITS7	GTGARTCATCGARTCTTTG	Ihrmark <i>et al.</i> (2012)
fITS7	GTGARTCATCGAATCTTC	Ihrmark <i>et al.</i> (2012)
ITS4Tul	CCGCCAGATTCACACATTGA	Taylors and McCormick (2008)
ITS1-OF	AACTCGGCCATTTAGAGGAAGT/AACTTGGTCATTAGAGGAAGT	Taylors and McCormick (2008)
ITS4-OF	GTTACTAGGGGAATCCTTGTT	Taylors and McCormick (2008)
ITS86F	GTGAATCATCGAATCTTTGAA	Turenne <i>et al.</i> (1999)
ITS1F	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns (1993)
ITS4B	CAGGAGACTTGTACACGGTCCAG	Gardes and Bruns (1993)
ITS1	TCCGTAGGTGAACCTGCGG	White <i>et al.</i> (1990)
ITS2	GCTGCGTTCTTCATCGATGC	White <i>et al.</i> (1990)
ITS3	GCATCGATGAAGAACGCAGC	White <i>et al.</i> (1990)
ITS4	TCCTCCGCTTATTGATATGC	White <i>et al.</i> (1990)
NS7	GAGGCAATAACAGGTCTGTGATGC	White <i>et al.</i> (1990)
cNL2f	GTTTCCTTTTAACAATTTAC	White <i>et al.</i> (1990)

Table 4 - List of recommended primer pair for sequencing of orchid mycorrhizal partners

Primer pair	Primer name (forward/ reverse)	Sequence (5'-3')	Target region	Annealing temp (°C)	Target clade (orchid specific group fungi)	References
ITS1/ITS4	ITS1 (F) ITS4 (R)	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	ITS1/ITS2	53	All Basidiomycota	White et al. (1990)
ITS1/ ITS4- Tul	ITS1 (F) ITS4-Tul	TCCGTAGGTGAACCTGCGG CCGCCAGATTACACATTGA	ITS1/ITS2	54	<i>Tulasnella</i>	Taylor and McCormick (2008)
ITS1-OF/ITS4-OF	ITS1-OF (F) ITS1-OF (F) ITS4-OF (R)	AACCTCGGCCATTTAGAGGAAGT AACTTGGTCATTTAGAGGAAGT GTTACTAGGGGAATCCTTGTT	ITS1/ITS2	60	All Basidiomycota	Taylor and McCormick (2008)
SSU1318-Tom/LSU-Tom4	SSU1318-Tom (F) LSU-Tom4	CGATAACGAACGAGACCTTAT GCCCTGTTCCAAGAGACTTA	SSU/LSU	62	Thelephoraceae	Taylor and McCormick (2008)
ITS86F/ITS4	ITS86F (F) ITS4 (R)	GTGAATCATCGAATCTTTGAA TCCTCCGCTTATTGATATGC	ITS-2	59	Both Ascomycota and Basidiomycota (including some <i>Tulasnella</i>)	White et al. (1990)
ITS3/ITS4OF	ITS3 (F) ITS4OF (R)	GCATCGATGAAGAACGCAGC GTTACTAGGGGAATCCTTGTT	ITS-2	62	All Basidiomycota	White et al. (1990) Taylor and McCormick (2008)
5.8S-Tulngs/ITS4-Tul2	5.8S-Tulngs ITS4-Tul2	CATTCGATGAAGACCGTTGC TTCTTTTCCTCCGCTGAWTA	ITS-2	57	All Basidiomycota (inc. Serendipitaceae and <i>Tulasnellaceae</i>)	Rammitu et al. (2021) Oja et al. (2014)
NS7/ITS1OF-RC	NS7 (F) ITS1OF-RC-G (R) ITS1OF-RC-A (R)	GAGGCAATAACAGGTCTGTGATGC ACTTCCTCTAAATGGCCGAGTT ACTTCCTCTAAATGACCAAGTT	SSU	62	Some Ascomycota and Basidiomycota (including some <i>Tulasnella</i>)	White et al. (1990) Waud et al. (2014) Waud et al. (2014)
ITS1OF/ITS2 m	ITS1-OF (F) ITS1-OF (F) ITS 2 m	AACCTCGGCCATTTAGAGGAAGT AACTTGGTCATTTAGAGGAAGT TCGCTGCGTTCCTCATCGA	ITS-1	62	Some Ascomycota and Basidiomycota (including some <i>Tulasnella</i>)	Taylor and McCormick (2008)
ITS1F/ITS2	ITS1F (F) ITS2 (R)	CTTGGTCATTTAGAGGAAGTAA GCTGCGTTCCTCATCGATGC	ITS-1	62	Both ascomycota and basidiomycota (including some <i>Tulasnella</i>)	Gardes and Bruns (1993) White et al. (1990)
ITS4OF-RC/cNL2F	ITS4OF-RC (F) cNL2F (R)	AACAAGGATTCCTTAGTAAC GTTTCCTTTTAAACAATTTCAC	LSU	59	Some Ascomycota and Basidiomycota (including some <i>Tulasnella</i>)	Waud et al. (2014) White et al. (1990)

Table 5 - List of endophytic fungi that has been isolated and identified from root by using a broad-spectrum primer (ITS1 and ITS4)

Orchid Species	Endophytic fungal (Accession no./taxonomic affiliation)	Type of primer	Country	References
<i>Vanda wightii</i>	<i>Ceratobasidium</i> _Wyd1 (MW59578)	ITS1 and ITS4	India	Suresh <i>et al.</i> (2023)
<i>Dendrobium longicornu</i>	<i>Alternaria</i> sp. (MN256650), <i>Cladosporium</i> sp. (MN256649), <i>Coniochaeta</i> sp. (MK225602), <i>Penicillium</i> sp. (MN256653), <i>Fusarium</i> sp. (MN256645), <i>Fusarium</i> sp. (MN256647), <i>Fusarium</i> sp. (MN256646).	ITS1 and ITS4	Nepal	Shah <i>et al.</i> (2022)
<i>Aerides rosea</i>	<i>Tulasnellaceae</i> sp. (JF691200)	ITS1 and ITS4	China	Zhao <i>et al.</i> (2021)
<i>Dendrobium nobile</i>	<i>Tulasnella deliquescens</i> (LC175331)	ITS1 and ITS4	China	Zhao <i>et al.</i> (2021)
<i>Dendrobium cucullatum</i>	<i>Tulasnella</i> sp. strain SSCDO-4 (MH348613)	ITS1 and ITS4	China	Zhao <i>et al.</i> (2021)
<i>Epigeneium amplum</i>	<i>Tulasnella</i> sp. 140 (AY373281)	ITS1 and ITS4	China	Zhao <i>et al.</i> (2021)
<i>Gastrochilus calceolaris</i>	<i>Ceratobasidium</i> sp. GC (GQ369961), <i>Ceratobasidium</i> sp. FPUB 168 (EF536969), <i>Rhizoctonia</i> sp. Abn1b (AJ318432), <i>Rhizoctonia</i> sp. Onv6 (AJ318436)	ITS1 and ITS4	Bangladesh	Hossain (2019)
<i>Aerides multiflora</i>	<i>Ceratobasidium</i> sp. (JX913820), <i>Ceratobasidium</i> sp. (JX913820),	ITS1 and ITS4	India	Bhatti <i>et al.</i> (2017)
<i>Paphiopedilum villosum</i> (Lindl.) Stein.	<i>Tulasnella</i> sp. (AY373281)/ <i>Tulasnellaceae</i> <i>Rigidoporus vinctus</i> (HQ400710)/ Polyporales <i>Ceratobasidium</i> sp. (HM117643)/ <i>Tulasnellaceae</i> <i>Flavodon</i> <i>flavus</i> (JQ638521)/Polyporales <i>Nigroporus</i> <i>vinosus</i> (AB811859)/Polyporales <i>Corioloopsis retropicta</i> (KC867403)/Polyporales <i>Valsa eugeniae</i> (AY347344)/Diaporthales	ITS1 and ITS4	Thailand	Khamchatra <i>et al.</i> (2016)
<i>Aerides multiflorum</i>	<i>Ceratobasidium</i> sp. (Eu605733)	ITS1 and ITS4	western Himalayas	Hossain <i>et al.</i> (2013)
<i>Rhynchostylis retusa</i>	<i>Ceratobasidium</i> sp. (Eu605732)	ITS1 and ITS4	western Himalayas	Hossain <i>et al.</i> (2013)
<i>Pecteilis susannae</i> (L.)	<i>Epulorhiza</i> sp. GQ856216 <i>Epulorhiza</i> sp. GQ856215 <i>Epulorhiza</i> sp. GQ856214 <i>Fusarium</i> sp. GQ862347 <i>Epulorhiza</i> sp. FJ882028 <i>Epulorhiza</i> sp. GQ862346 <i>Epulorhiza</i> sp. FJ940903 <i>Epulorhiza</i> sp. FJ873174	ITS1 and ITS4	Thailand	Chutima <i>et al.</i> (2011)

(Taylor and McCormick 2008). To address this issue, the ITS4-Tul primer has been designed to study only *Tulasnella* species, thereby minimising the amplification of other taxa. Two primers that are *Tulasnella* specific which is ITS4-Tul and ITS4R are designed from the 3-end of ITS2 (Suárez *et al.*, 2006). The ITS4-Tul primer is a perfect or near-perfect match for some of the core species of *Tulasnella* but their mismatches with the majority of other fungi make them a specific primer. ITS4-Tul has been used widely as a primer, especially for the identification of orchid mycorrhizal primarily targeted *Tulasnellaceae*, which are mostly reported to have the ability to promote seed germination (Oja *et al.*, 2014; McCormick *et al.*, 2021; Suetsugu *et al.*, 2021). Meanwhile, ITS1-OF and ITS4-OF is nowadays are increasingly used in characterising orchid fungal symbionts as they are designed to be a broad spectrum basidiomycete specific primer (Currah and Sherburne, 1992; Taylor and McCormick, 2008; Jacquemyn *et al.*, 2010).

A study on identification of fungi identification of terrestrial orchid mycorrhizal by using broad spectrum fungal taxa primer (ITS86F/ITS4) by Waud *et al.* (2014) has outperformed the other primer pair. The study also assessed the efficacy of several type of broad-spectrum primer and specific primer for orchid mycorrhizal fungi to understand and characterized orchid mycorrhizal communities and suggested several suitable primer pairs. Other study also uses the broad-spectrum primer pair ITS86F/ITS4 to investigate the orchid mycorrhizal community in both epiphytic and terrestrial orchid (Cevallos *et al.*, 2017; Johnson *et al.*, 2021). However, the use of broad-spectrum primer for identification of orchid mycorrhizal fungi is constrained by a primer bias, which arise from the inability of the primer to identify a specific fungus within a sample due to the mismatch during PCR. While *Tulasnellaceae* fungi are commonly associated with orchids (Dearnaley *et al.* 2012), their molecular detection poses challenges due to mismatches with universal fungal primers (Suárez *et al.*, 2006; Taylor and McCormick 2008; Waud *et al.*, 2014; Rammitsu *et al.*, 2021).

Moreover, previous comprehensive investigations conducted through Sanger sequencing-based methodologies have indicated distinctions between the mycorrhizal communities associated with epiphytic orchids and those associated with terrestrial orchids (Martos *et al.*, 2012; Xing *et al.*, 2019). The utilising of *Tulasnellaceae*-specific primers

for the assessment of orchid mycorrhiza; networks by metabarcoding analysis is highly recommended, particularly in the context of epiphytic orchids, as emphasised in the research conducted by Rammitsu *et al.* (2021). The commonly used broad spectrum primer, ITS86F/ITS4 effectively identified *Ceratobasidiaceae* and *Serendipitaceae* fungi but proved inadequate in detecting the diversity of *Tulasnellaceae* fungi (Rammitsu *et al.*, 2021). Due to significant primer biases present within the *Tulasnellaceae* family, which plays a crucial role as mycorrhizal symbionts in the majority of orchid species, it is imperative to exercise caution in selecting primers and thoroughly assess potential biases (Oja *et al.*, 2014).

8. Sequencing

When it comes to fungi, morphology is often the method of choice for performing the fundamental function of species distinction. However, distinguishing species based on their morphology can be difficult, particularly for fungi that do not have complex fruiting bodies, as is the case with the three families of *Rhizoctonia* species that are linked with orchids (Gardes and Bruns, 1993). Conventionally, it has been thought that the 'Rhizoctonia' complex, which includes species from three different fungal families (*Tulasnellaceae*, *Ceratobasidiaceae*, and *Serendipitaceae*), makes up the bulk, if not the entirety, of orchid mycorrhizal fungus. However, recent research suggests that this may not be the case. Septal ultrastructure is a defining characteristic that separates the various clades within *Rhizoctonia* (Currah and Sherburne, 1992), but careful inspection is still required to distinguish *Sebacinaceae* and *Tulasnellaceae* (Andersen, 1996). This problem is compounded by the fact that when the cryptic, resupinate fruiting structures are seldom observed. Basidial morphology offers suitable identification of orchid-associated *Rhizoctonia* species at the morphospecies level (Warcup and Talbot, 1967). However, orchid isolates are rarely induced to fruit in culture as some fungi cannot be produced in artificial circumstances (Currah *et al.*, 1990).

In order to expand knowledge of fungal variety, culture-independent technologies (sequencing and cloning) have been created. Morphological identification methods are conventional identification method that involves evaluating the

morphological and microscopic features of fungi on different culture media and under different conditions. This method can be accompanied by other identification methods to help identify fungi more accurately. Other methods, such as microscopic examination or biochemical screening, can be performed alone or in conjunction with molecular analysis. With the recent development of advanced molecular techniques (e.g., next-generation sequencing), the spectrum of fungi discovered at the species level has expanded significantly, allowing for more precise ecological inferences (Peay, 2014).

High-Throughput Sequencing (HTS) technologies provide a number of benefits, including the capability to identify fungi at trace levels, quick microbial community structure analysis, and cost-effectiveness (Cruz *et al.*, 2014; Tedersoo and Nilsson, 2016; Nilsson *et al.*, 2019). These benefits can be found in HTS technologies. According to Nilsson *et al.* (2019), a typical HTS metabarcoding process consists of several important stages, including DNA extraction, marker-based PCR amplification, DNA sequencing, sequence processing, and data analysis. These processes are listed in the order as follows: sampling then DNA extraction. However, one potential downside of these technologies is that they may potentially result in the spread of pollutants and mycorrhizal fungi that are not specific to orchids.

Research methodology and sequencing carried out on high-throughput platforms are the two components of the most typical approaches to molecular identification. DNA microarrays, clone libraries, denaturing gradient gel electrophoresis, fluorescence *in situ* hybridization, and gene chip approaches are some of the other methods that can be utilised for the identification of fungi (Dearnaley, 2007). However, these technologies have shortcomings such as limited throughput, time-consuming processes, and lower accuracy. Additionally, they have been overshadowed by the growing popularity of alternative methods such as the MiSeq PE300 and HiSeq PE250 platforms (Julou *et al.*, 2005).

Furthermore, alternative methods, such as using an Illumina NovaSeq/HiSeq sequencer and the application of shotgun metagenomic technology, provide access to functional gene information from all microorganisms within a community through genomic DNA analysis (Bahram *et al.*, 2018; Fadiji and Babalola, 2020). These methods were developed by Bahram *et al.* (2018) and Fadiji and Babalola (2020). An important step forward in orchid mycorrhiza

research has been taken thanks to the development of this technique and the growing availability of orchid and reference orchid mycorrhizal fungal genomes (Zhang *et al.*, 2016).

Because of their ability to simultaneously sequence a mixed DNA template across numerous samples with a high sequencing depth (Nilsson *et al.*, 2019), next-generation sequencing (NGS) approaches have become practically widespread in mycorrhizal research in recent years. This is partly owing to the fact that NGS methods have grown more affordable in recent years. In contrast, sequencing DNA from individual mycorrhizal root tips may be ideal for Sanger sequencing when it comes to detecting shifts in regularly occurring fungus species (Shemesh *et al.*, 2020). This was found by Shemesh and colleagues. In contrast to next-generation sequencing technologies, which can process millions of DNA fragments simultaneously, the Sanger sequencing method only processes one DNA fragment at a time (Slatko *et al.*, 2018). This makes the Sanger sequencing method superior in terms of sequencing volume. This distinction has the ability to bring forth different conclusions regarding the make-up of the community.

9. Conclusions

This review provides an overview of the most significant literature in orchid mycorrhizal fungi from about 2002-2023. The molecular identification of orchid mycorrhiza represents a significant advancement in our understanding of the complex relationships between orchids and their mycorrhizal fungal. In addition, finding the most appropriate extraction method and choosing a suitable primer for amplification is essential to ensure accurate identification. Moreover, the utilization of molecular techniques compliments morphology-based identifications offers a reliable, unbiased, and frequently more precise tools for confirming species. It is particularly beneficial for cryptic species, hybrids, morphological variables organism such as mycorrhizal, or situations when usual identification methods fail. Based on the review, the ITS regions prove to be a great primer in the field of mycorrhizal studies to its inherent variability, widespread applicability and straightforward amplification process and compatibility with established databases. This technique enables researchers to accurately identify the specific fungal species

associated with a particular orchid species and to investigate the functional role of these fungi in orchid growth and development. With the advancement of molecular techniques, it is now possible to examine the genetic diversity of these fungi and understand the evolutionary relationship between different orchid mycorrhizal fungi. These may lead to the development of new conservation strategies for these unique and valuable plant species.

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