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Research Article

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Unveiling Fungal Relationships in Ophrys Species: A Molecular Perspective

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Abstract In Turkey, orchids face a grave risk of extinction due to a combination of excessive harvesting and the severe degradation of their natural habitats. Research has revealed that safeguarding orchids hinges on the diversity of mycorrhizal fungi. This study aims to identify orchid mycorrhizal fungi from the root systems of nine different Ophrys species originating from Muğla province in Turkey. After conducting molecular identification, the objective is to establish a repository of these fungi. To achieve this, plant roots from Ophrys species were collected during the spring and summer months between 2018 and 2020. The ITS1-5.8S-ITS4 region was subsequently amplified using ITS4 and ITS1 primers. DNA sequencing revealed that out of the ten Rhizoctonia-like isolates, eight were associated to sequences belonging to Ceratobasidaceae species, while two exhibited associations with sequences from Tulasnellaceae species.

Keywords Ceratobasidium, Ophrys, Orchid mycorrhizal fungi, Symbiosis, Tulasnella

1. Introduction

For more than a century, it has been recognized that members of the Orchidaceae family form mycorrhizal associations with Basidiomycota [1] and Ascomycota [2, 3] members. Orchid seeds are minute and lack the necessary nutrients for initiating germination. As a result, the initial phase of seedling growth relies on mycorrhizal interactions. In their natural habitat, these seeds remain dormant unless in the presence of a suitable fungus.

Orchid Mycorrhizal fungi (OrM) are primarily beneficial to plants by improving their nutrient access and stress tolerance. As a result, they play a significant role in regulating plant populations and community biology by controlling seedling establishment and species coexistence. For orchids, in particular, mycorrhizal associations are critical, as most orchids rely entirely on mycorrhizal fungi for seed germination [4, 5, 6]. Multiple investigations have indicated the transfer of carbohydrates from the mycobionts to the maturing orchid plant. However, there has been inconsistent evidence regarding the consistent demonstration of carbon transportation in any configuration, from orchid to fungus [7].

There is evidence to suggest that these mycorrhizal associations could significantly impact the local orchid population by enabling them to adapt to various physiological changes during seedling development [8, 9]. The primary determinant influencing seedling establishment is the level of specificity between orchids and fungi [10]. Essentially, mycorrhizal fungi play an indispensable role in the orchid life cycle. However, there remains limited knowledge about the diversity of these mycorrhizal fungi [11, 12]. As a result, it can be proposed that the presence and abundance of orchids within their habitats are closely linked to the distribution and prevalence of their mycorrhizal fungi, as suggested by various studies [8, 11, 12, 13]. Therefore, to ascertain the spread of orchid fungi, two methods are employed. The initial approach involves extracting fungi from orchid roots, while the second method entails burying seed packages in soil [14]. The fungi obtained through isolation have been

characterized using both morphological and molecular methods [15,16]. Nevertheless, a comprehensive understanding of the direct relationship between OrM and orchid distribution remains incomplete.

In Turkey, there is a notable presence of approximately 200 orchid species. All orchids in Turkey, whether native or not, are subject to legal protection. Despite the prohibition on collecting bulbs, there is an excessive annual collection due to their utilization in salep and ice cream production [17].

Conversely, there exists a dearth of comprehensive research concerning the physiological aspects of orchid seed germination and the diversity of mycorrhizal fungi. These gaps are crucial for both fostering production and ensuring protection. To facilitate orchid cultivation and conservation efforts, it is imperative to first identify the mycorrhizal fungi specific to these species, followed by the establishment of an orchid fungus bank.

Building upon our earlier research endeavor focused on this matter, certain species were examined to identify their mycorrhizal fungi [18, 19]. In our ongoing investigation, we are extending our exploration in this area. The objective of our current study is to provide insights into the relationship between the range of mycorrhizal fungi present in *Ophrys* species and their respective environments. Additionally, we aim to secure fungal specimens that can be employed in forthcoming studies involving soil-based seed production. The collected fungal materials will also be incorporated into the orchid fungus repository.

2. Materials and Methods

Orchid species

Sampling was performed in Muğla province in Turkey to discover the mycorrhizal fungus associated with orchids. 9 *Ophrys* (*Ophyrs iricolor, Ophyrs fusca, Ophrys mammosa, Ophyrs leucadica, Ophyrs holoserica, Ophyrs sphegodes, Ophyrs umblicata, Ophyrs apifera,* and *Ophyrs oestifera*) species were identified following the guidelines outlined in the Flora of Turkey [20]. Healthy plant roots were collected while the plants were in the flowering stage, specifically during the spring and early summer of 2018 and 2020. Five randomly chosen individual plants from a single population of each orchid species were selected, and two root samples were gathered from each of these orchid plants.

Isolation of fungal symbionts

To ascertain the traits of peloton-forming fungi present in orchid roots, the fungi were isolated [21]. For this, the roots underwent surface sterilization by immersing them in a 1,5% NaOCl solution for 1-3 minutes, followed by rinsing in distilled water before being placed (on set) in the agar medium. Subsequently, the Petri dishes were placed within a laminar flow system and then incubated at a consistent temperature of 25±2ºC. The developing fungal hyphae were extracted from the roots following the procedure outlined in [22]. Microscopic examination of the fungal hyphae was conducted using a light microscope (Leica microscope). The quantification of nuclei per cell was also executed [23].

DNA extraction, PCR amplification and Phylogenetic tree construction

Fungal culture-derived DNA extraction was done from 0.5 g mycorrhizal root using the Cetyltrimethyl Ammonium Bromide (CTAB) method [24]. PCR amplification was conducted using the subsequent cycling conditions: initial denaturation at 94°C for 4.5 minutes, succeeded by 33 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 51°C for 30 seconds, and extension at 72°C for 45 seconds. PCR reactions were executed using the universal ITS1 and ITS4 primers [25]. Macrogen (Korea) conducted sequencing of the amplicons derived from the fungal isolates using an ABI 3730 XL sequencer. Sequencing primers ITS1 and ITS4 were employed, and the amplicons were sequenced bidirectionally.

The sequences were edited and combined utilizing the MEGA 6 software program [26]. For sequence identification, the BLASTn algorithm provided by the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) was employed to compare against the NCBI nucleotide collection.

3. Results

Fungal isolates yielded rDNA gene regions comprising ITS1–5.8S–ITS2 upon running, resulting in product lengths ranging from 500 to 700 bp as observed on electrophoresis. Subsequently, serial analysis of the ITS1–

5.8S–ITS2 gene region products derived from the isolates, amplified with ITS1 and ITS4 primers, was conducted in collaboration with Macrogen Company. Consensus sequences were generated using the Sequencher 4.7 Demo software. These consensus sequences were then compared to sequences stored in the Genebank (NCBI) through blast analysis.

Totally 10 fungi were isolated from the roots of the *Ophrys* species: one fungal strain from *O.fusca*, *O.mammosa*, *O.leucadica*, *O.holoserica*, *O.sphegodes*, *O.umblicata*, *O.apifera*, and *O.oestifera* and two fungal strains were isolated from *O. iricolor*. The closest matches identified through BLAST searches have been detailed in Table 1. Ceratobasidiaceae fungi (basidiomycetes) were found to be associated with the majority of the orchids, precisely eight out of ten samples with 80-99% resemblance. The remaining sequences were associated with Tulasnellaceae fungi (basidiomycetes) with 93–95% resemblance.

Table 1: Molecular identification in GenBank based on the closest match of 10 mycorrhizal fungi isolated from the root of *Ophrys* species. Isolate ID, plant source, the Genbank Accession Number, DNA base length (bp) and the Accession Numbers of fungal sequences with the closest matches according to the Blast result with the highest percentages of matching and their references

Isolate ID	Plant Source	Genbank (NCBI) Accession No.	Lengh (bp)	Resembl ance (%)	Closest match with GenBank	References
Op1	*Ophyrs iricolor*	OR607750	523	90	*Ceratobasidium* sp. (MF407555)	Bruzone et al. 2017
Op2	*Ophyrs fusca*	OR610686	603	95	Uncultured *Tulasnella*(JF926482)	Girlanda et al.2011
Op3	*Ophrys mammosa,*	OR589279	540	94	*Ceratobasidium* sp. (KX611566)	Bruzone et al. 2017
Op4	*Ophyrs leucadica*	OR589435	477	99	Uncultured *Ceratobasidium* (JF912463)	Ercole et.al 2011
Op5	*Ophyrs holoserica*	OR602794	485	90	*Ceratobasidium* sp. (HM117643)	Paduano et.al 2011
Op6	*Ophyrs sphegodes*	OR589436	550	96	*Ceratobasidium* sp. (HM117643)	Paduano et al.2011
Op7	*Ophyrs umblicata*	OR610685	747	85	Uncultured Ceratobasidiaceae (KC243941)	Tesitelova et.al 2013
Op8	*Ophyrs iricolor*	OR608229	682	80	*Rhizoctonia* sp. (HQ738654)	Guermache et al., 2012
Op9	*Ophyrs apifera*	OR589442	849	93	*Rhizoctonia fraxini*(MH855687)	Vu et.al 2019
Op10	*Ophyrs oestifera*	OR610874	750	93	Uncultured Tulasnellaceae (KJ188462)	Tesitelova et.al 2015

Based on the phylogenetic tree, OP1 is associated with Uncultured *Ceratobasidium* (FJ5688129.1). OP3 is associated with Uncultured *Ceratobasidium* (KJ716224.1). OP4, OP5, and OP6 are associated with *Ceratobasidium* sp. (MH117643.1). OP 7 sequence is found to be closely associated with the clad Uncultured Ceratobasidaceae (LC440230.1) and *Rhizoctonia solani* AG I (MT177265.1, JQ59888.1, and KX964588.1 respectively). OP8 was associated with *Rhizoctonia bicornis* (MN265834.1) and OP9 was clustered with *Ceratobasidium* sp. (MG762693.1) (Figure 1). Regarding the other two sequences associated with Tulasnellaceae. Finally, OP2 is associated with Uncultured Tulasnellaceae (KC243935) and Tulasnellaceae sp. (OR50712.1) and OP10 was clustered with Uncultured Tulasnellaceae (KJ188462.1) (Figure 2).

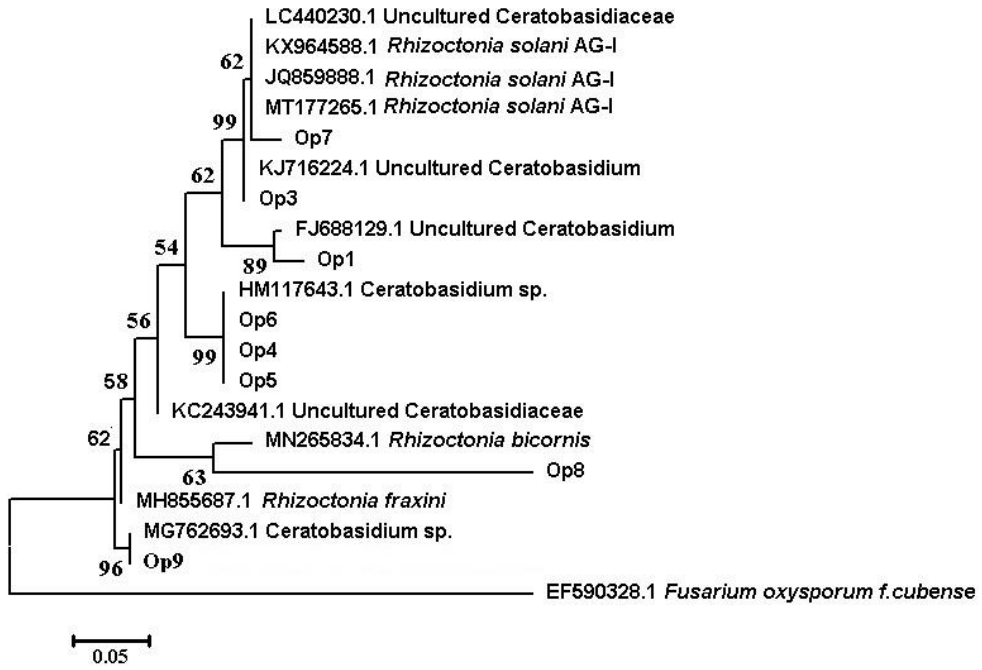

Figure 1: Maximum Likelihood tree showing the phylogenetic relations of 5.8S rDNA ITS nucleotide sequences of Ceratobasidaceae fungi obtained in this study. On the tree, bootstrap values greater than 50% have been shown, as have the bootstrap values of ML.

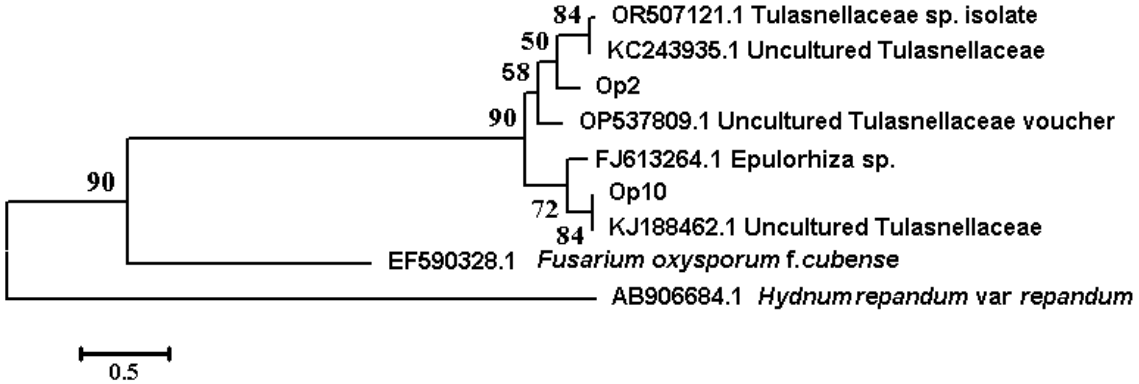

Figure 2: Maximum Likelihood tree showing the phylogenetic relations of 5.8S rDNA ITS nucleotide sequences of Tulasnellaceae fungi obtained in this study. On the tree the bootstrap values greater than 50% have been shown and the bootstrap values of ML.

4. Discussion

Turkey boasts a significant wealth of orchid species. However, the excessive harvesting of bulbs for salep and ice cream production poses a serious threat to these orchids, potentially leading to their extinction. In nature, the primary mechanism facilitating orchid propagation from seeds involves mycorrhizal fungi. Unfortunately, comprehensive research on orchid seed germination and mycorrhizal fungal diversity is notably scarce in Turkey. While some studies have explored the morphological and molecular characteristics of fungi involved in mycorrhizal associations with specific *Dactylorhiza* species [18] and *Orchis* species [19] in Turkey, there remains a dearth of research regarding the molecular characterization of other orchid species and genera.

The sequence data of fungi reproduced from the ITS1-5.8-ITS2 gene of identified fungi of *Ophrys* species revealed the presence of various fungal taxa within the root samples. Notably, Basidiomycota was consistently

detected across all orchid samples (Table 1). Hence, it becomes imperative to perform molecular identification [27,28]. In a parallel research effort carried out in Brazil [29] showed that it is essential to conduct molecular characterization of fungi isolated from the roots of *Epidendrum scendrum* to demonstrate their association with distinct Tulasnellaceae and Ceratobasidaceae species.

These findings indicate that the orchid species under investigation likely form symbiotic associations with a prevailing fungal partner belonging to the Tulasnellaceae and Ceratobasidaceae family. In our study, mycorrhizal fungi from 4 *Ophrys* species (*O.iricolor, O.mammosa, O.holoserica,* and *O.sphegodes*) found the best similarity in BLAST searches with *Ceratobasidium* sp. from populations of *Pterostylis revoluta* [30] and *Limodorum abortivum* [31]. Additionally, mycorrhizal fungi from *O. fusca* and *O.oestrifera* found the best similarity in BLAST searches with Uncultured Tulasnellaceae from populations of *Ophrys fuciflora* [32] and *Neottia cordata* [33]. The remaining 2 Fungal taxa from *O.iricolor* and *O.apifera* found the best similarity in BLAST searches with *Rhizoctonia* sp. [34,35].

Our results align with several recent studies that have reported the presence of Tulasnellaceae and Ceratobasidaceae fungi in various orchids, particularly those belonging to photosynthetic terrestrial species, found in diverse habitats including forests [36] and open environments [32,37].

Similarly, a recent research conducted a comprehensive analysis of fungi in *Ophrys fuciflora* roots, employing both culture-dependent and culture-independent ITS sequence analysis methods [32]. The findings revealed a significant association with *Tulasnella* and *Ceratobasidium*, with an occurrence rate of 40%, whether assessed through direct DNA extraction from mycorrhizal roots or fungal isolation.

5. Conclusion

Our research represents the initial comprehensive investigation into the diversity of mycorrhizal fungi linked to *Ophrys* species in Turkey. The scope of the inaugural orchid fungus repository established in Turkey, which encompasses the isolation and storage of fungi engaged in mycorrhizal partnerships, will expand in the future as additional studies in this area are undertaken.

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