ISOLATION AND IDENTIFICATION OF MYCORRHIZAL FUNGUS FROM AN EPIPHYTIC ORCHID (*RHYNCHOSTYLIS RETUSA* L. BL.)

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Abstract

Isolation and identification of mycorrhizal fungi from the roots of *Rhynchostylis retusa* indicated that the cultural and microscopic features, namely colony appearance, colony colour, diameter of vegetative hyphae, presence of monilioid cells, right angle branching pattern of the fungal endophyte corroborated the identity of the fungus *Rhizoctonia* like anamorphs of *Ceratobasidium* species. Fungal identity was further confirmed through sequencing and analysis of internal transcribed spacer (ITS) sequences of the nuclear ribosomal DNA (nrDNA). ITS sequence of the isolate RhY10A6 (accession no. MN120903.1) showed > 99% similarity with several isolates of the teleomorphic fungus *Ceratobasidium* sp. in NCBI mega blast search. The phylogenetic analysis based on maximum parsimony method, this orchid mycorrhizal fungus clustered with several isolates of *Ceratobasidium* sp. or *Rhizoctonia* like fungi. It showed near distant relation with *Ceratobasidium ramicola* (GeneBank accession no. NR_138368.1) which is an orchid mycorrhizal fungus. Therefore, molecular characterization validated the morphological data. The techniques established in this orchid will facilitate to isolate and accurate identification of mycorrhizal fungus.

Introduction

In nature, one of the most ubiquitous interactions involving orchid roots and certain heterogeneous group of fungi is termed as orchid mycorrhiza. Orchids are solely dependent on mycorrhizal fungi for seed germination, growth and establishment in nature (Arditti *et al.* 1990). Orchids are categorized into three groups based on mycorrhizal dependency as mycoheterotropic, holomycotropic and mixotropic (Dearnaley 2007). All orchids are mycoheterotrophic during their early stages of development and most of the cases the relationship exists throughout the life. Study of orchid mycorrhiza is very important in terms of their evolutionary and adaptive significances, role in orchid speciation and conservation procedures (Dearnaley *et al.* 2012, Ruibal *et al.* 2017). Orchid mycorrhizal fungi have the potential to improve the sustainability of commercial orchid cultivation by reducing the need for costly and environmentally damaging chemical inputs such as fertilizers and pesticides because the fungi are eco-friendly, enhance nutrient uptake by plants and increase resilience to environmental stresses (Suz *et al.* 2018). Isolation and identification of mycorrhizal fungi is the first step of its applications for commercial orchid propagation and conservation (Zhang and Zhou 2004). Compatible mycorrhizal fungi are proficient to stimulate seed germination, enhance the growth of protocorms, young seedlings or juvenile plants, and improve *ex vitro* survivability of tissue culture raised seedlings (Hossain *et al.* 2013).

From the groundwork of orchid mycorrhiza, *Rhizoctonia* spp. were considered to be the only fungal partner associated with orchids. Later on, many fungi identified from orchids showed resemblance in many aspects to *Rhizoctonia* hence are collectively called *Rhizoctonia*-like fungi (Shan *et al.* 2002, Sharon *et al.* 2008). The *Rhizoctonia* species associated with orchids include free-living saprophytes and opportunistic soil pathogens.

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The systematics of orchid mycorrhizal fungi were studied using both morphological (Shan *et al.* 2002, Jakucs *et al.* 2005, Zhu *et al.* 2008), and molecular characters (Taylor and McCormick 2008, Sharon *et al.* 2008, Thakur *et al.* 2018). Traditionally the *Rhizoctonia*-like fungi are characterized based on the cultural morphology, cytomorphology of the hyphae, monilioid cells, teleomorphs, number of nuclei in the cells. These features are studied by applying several staining methods such as aniline blue or trypan blue, HCl-Geimsa, orcein, and acridine orange (Shan *et al.* 2002).

The conventional approaches to identify orchid mycorrhizal fungi have limitations as most of the fungi associated with orchids are mycelia sterilia. Consequently, the broad vegetative criteria for identification have resulted in paraphyletic taxonomy, with various unrelated fungi being grouped together. These limitations necessitate the application of molecular techniques for accurate identification (Shan *et al.* 2002, Otero *et al.* 2004, Dearnaley 2007). ITS region sequencing of nrDNA is the common and powerful molecular technique for accurate identification of orchid mycorrhizal fungi (Kristiansen *et al.* 2001, Sharon *et al.* 2008, Jacquemyn *et al.* 2011). It provides a novel and very powerful DNA fingerprinting technique for DNAs of any origin or complexity. The present investigation was undertaken with a view to accurate identification of root associated mycorrhizal fungus in *R. retusa* following morpho-molecular techniques.

Materials and Methods

Fresh roots of *Rhynchostylis retusa* growing naturally in the Kaptai forest of Rangamati district of Chattogram, Bangladesh were collected in air-tight plastic bags during May-June (rainy season) and November-December (winter season) and used within 24 hrs for isolation of fungal symbionts. The root samples were taken randomly from five plants, washed with running tap water, cut thin transverse sections at different root portions, stained with lactophenol cotton blue and observed under microscope to ensure the presence of fungal colonization. The incidence of fungal colonization and formation of pelotons in the root sections was calculated by following formula (Hossain 2019):

$$\frac{\text{Number of cells colonized 20x microscope field view}}{\text{Total number of cells 20x microscope field view}} \times 100$$

The roots showing the presence of fungal pelotons were rinsed with distilled water and surface sterilized by 0.1% $HgCl_2$ for 6 - 10 mins and then washed 3 times with double sterile distilled water. Final disinfection was done by dipping them in 70% ethanol for 30 sec and washed 3 times with double sterile distilled water. The roots were then aseptically cut into sections approximately 2 mm thickness and placed in 9 cm Petri dishes containing potato dextrose agar (PDA) medium. The plates were incubated in the dark at 27°C until fungal hyphae were visibly growing from root specimens onto the medium. The fungi grown from the inner portion of the root section were considered probable mycorrhizal fungi. Pure cultures were obtained by transfering hyphal tips onto fresh PDA medium.

Nature of fungal growth, colony surface and reverse colours were recorded at young and mature stages. The hyphal diameter and dimensions of monilioid cells were measured by Nikon E600 light microscope (Nikon, Tokyo, Japan) by mounting the mycelium in lactophenol triglycero-cotton blue on glass slides. Growth rates were determined according to Currah *et al.* (1987) by inoculating uniform bits of mycelium at the middle of PDA plates. Radial increments in colony size were measured at 48 hrs interval over two weeks. Growth rates were represented by averages based on three replications.

For determination of nuclei number in vegetative and monilioid cells, a small portion of the mycelial mat was fixed in 2% formaldehyde for 2 min on a glass slide and rinsed with distilled water for 1 min, followed by staining with gold antifade reagent with diamidino-2-phenylindole (ProLong® DAPI, Invitrogen Ltd., Eugene, OR, USA) for 10 min, and destained with distilled water for 2 min. A drop of 50% glycerin was placed over the stained specimen and covered with cover slip. Micrographs were taken using Nikon E600 microscope equipped with fluorescence accessory with mercury lamp.

The fungal isolates were inoculated on sterilized potato dextrose agar medium (50 ml/250 ml flask) and incubated at 28°C in an incubator (New Brunswick Scientific, Edison, NJ) for two weeks. DNA was extracted from 15 days old cultures according to the protocol described by Liu *et al*. (2000).

The amplicons of ITS1, 5.8S ribosomal RNA and ITS2 were achieved using ITS1 (5' TCC GTAGGTGAACCTGCGG) and ITS4 primers (5'GCTGCGTTCATCGATGC) (White *et al*. 1990). The PCR reaction was performed in 50 µl reaction buffer containing 5 µl of dNTP mixture, 5 µl of 10×PCR buffer, 1 µl of each primer (10 pmol), 0.4 µl of Taq polymerase 1U, 2 µl of genomic DNA and 35.6 µl of MiliQ water. The PCR cycling condition comprised of an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec and extension at 72°C for 7 min. The amplified PCR products were purified and concentrated. The nucleotide sequences of the amplicons were generated at McLab, California, USA on a 3730 DNA Analyzer (Thermo Fisher Scientific, USA). A consensus sequence was generated from the forward and reverse sequences by using BioEdit 7.2.6 software (Hall 1999). The consensus sequence was deposited in the NCBI GenBank (Accession no. MN120903.1)

The consensus sequence was placed into web based Basic Local Alignment Search Tool (BLAST) of NCBI GenBank (https://blast.ncbi.nlm.nih.gov/) to find out similar species/isolates. The phylogenetic relationships were established based on maximum parsimony tree by using analysis program of the MEGA 6.0 software (Tamura *et al*. 2013). In this analysis Bootstrap replications number was 1000.

Results and Discussion

The fungal endophytes showed intra- and intercellular colonization of the cortical cells with peloton formation within the mature roots of *R. retusa* collected from their natural habitat (Fig. 1A). Colonization was observed only in the roots attached with substratum and no pelotons were found in the root tip region. Pelotons in the outer-cortex appeared with dense live hyphal coils while the inner cortex the pelotons showed brownish and loose interweaving (Fig. 1B,C). The incidence of fungal colonization in *R. retusa* was higher during June - July i.e. rainy season (94.74 ± 1.77%) than November-December i.e. winter season (74.29 ± 1.09%). Seasonal variation of fungal colonization in orchid roots was reported by Hossain *et al*. (2013). Higher colonization during the summer and rainy season was due to active vegetative growth of plant and flowering as compared to the winter season of slow growth. The observation also corroborated the earlier reports of higher colonization by compatible fungi to meet up the excessive nutrient demand for active vegetative growth and phenology of orchids (Masuhara and Kutsuya 1992, Shagufta *et al*. 1993). The brownish colour and loose hyphal coils displayed by the pelotons in the inner-cortex could be due to the digestion of fungal mass by the lytic enzymes produced by the host for discharging nutrients. Orchids also exhibit moderate defense mechanisms to control the level of fungal infection through the production of phytoalexin or orchinol that control the level of infection (Rasmussen and Whigham 2002).

Morphological features of the fungal endophytes isolated from *R. retusa* are summarized in Table 1. The colony morphology and microscopic features showed resemblance to *Rhizoctonia-*

like fungi, *Ceratobasidium* sp. The young colonies were cottony white and light brown at maturity (Fig. 1D) and brownish black on reverse with a slight constriction and a dolipore septum at the branching point of the hypha (Fig. 1E). The monilioid cells were nearly spherical in the fungal-isolate from *R. retusa* (Fig. 1F). Growth rate was 0.36 mm/hr. both vegetative and moniloid cells of the fungal-isolate were binucleate (Fig. 1E). The fungal endophytes isolated from *R. retusa* in the present studies showed peloton formation and monilioid cells (asexual resistant propagules)

Table 1. Morphological features of *Ceratobasidium* sp. isolated from *Rhynchostylis retusa*.

Parameters	Characteristics
Colour of young colony (surface)	Cottony white
Colour of young colony (reversed)	Brownish white
Colour of mature colony (surface)	Light brown
Colour of mature colony (reversed)	Brownish black
Colony appearance	Flat or felted
Colour of vegetative hyphae	Hyaline
Diameter of vegetative hyphae	7 - 12 μm
Shape of monilioid cells	Nearly spherical
Diametre of monilioid cells	12 - 15 μm
Branching pattern	Right angle with slight constriction at the branching point
Colony growth rate (mm/hr)	0.36
Nuclear condition	Binucleate
Teleomorph	Absent

development which are the common features for *Rhizoctonia*-like orchid endophytes (Rasmussen and Whigham 2002, Shan *et al.* 2002). Fungi placed in the form-genus *Rhizoctonia* seldom reveal their basidiocarps but produce monilioid cells which are the resistant asexual anamorphic feature common to these fungi. Nuclear number per cell varies from uninucleate, binucleate to multinucleate in different orchid mycorrhizal fungi and even in different strains of the same species is another important feature for classification of *Rhizoctonia*-like fungi (Otero *et al.* 2002). Uninucleate *Rhizoctonia*-like fungi considered pathogenic for other plants are rarely found in orchids (Otero *et al.* 2002, Rasmussen and Whigham 2002). Multinucleate *Rhizoctonia*-like fungi were also reported in orchids (Masuhara *et al.* 1993, Carling *et al.* 1999, Hossain 2019). The endophytes in the present studies with two nuclei in vegetative cells and monilioid cells belonged to the binucleate group of *Rhizoctonia*-like fungi which are reportedly most common and broadly distributed in orchids (Otero *et al.* 2002).

In the results of NCBI BLAST search, 12 different isolates of *Ceratobasidium* sp. and 1 isolate of *Rhizoctonia* sp. showed > 99% ITS sequence similarity, 99% query coverage, 0.0 e-value and 1195 - 1179 score with the mycorrhizal isolate RhY10A6 (MN120903.1). Three type species, *viz.*, *Ceratobasidium ramicola* CBS 133.82, *Ceratobasidium papillatum* CBS 570.83 and *Ceratobasidium angustisporum* CBS 568.83 showed 84 - 88% ITS sequence similarity, 85 - 86% query coverage and 527 - 676 score with the mycorrhizal isolate. According to Singh *et al*. (2013) ≥ 95% query coverage and ≥ 99% similarity were considered as same species in sequence-based

identification. Therefore, the mycorrhizal isolate RhY10A6 was identified as a teleomorph *Ceratobasidium* sp. of anamorph *Rhizoctonia* sp. on the basis of ITS sequence analysis (Fig. 2).
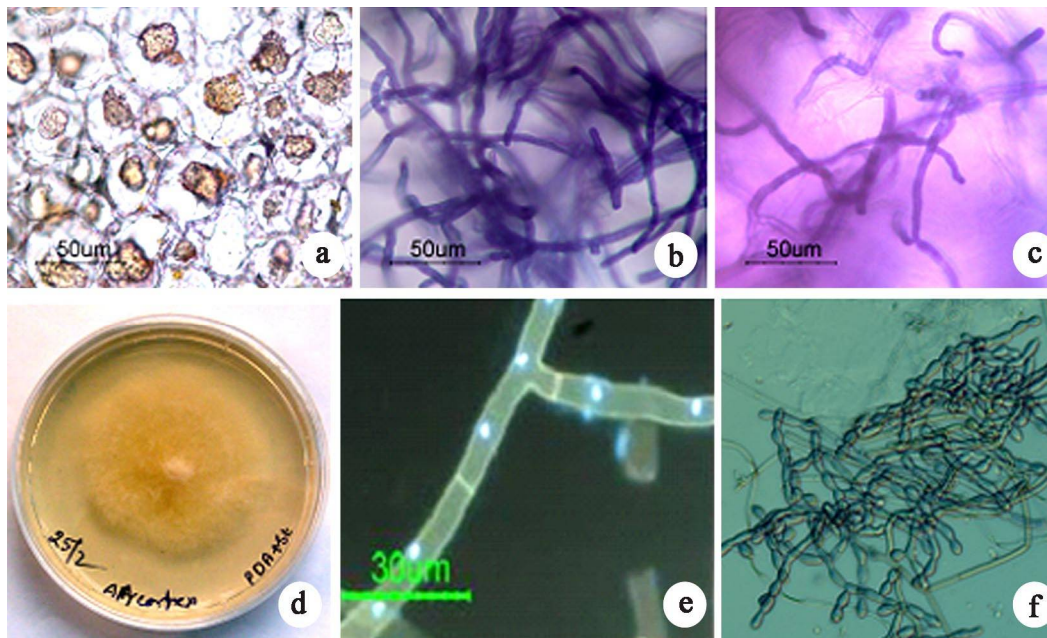

Fig. 1. Morphological characteristics of the fungal endophyte: a. The fungal pelotons in the mature roots sections of *R. retusa*; b. Dense live hyphal coils appeared in the outer-cortex; c. Loose hyphal coils in the inner cortex; d. A mature fungal colony; e. Binucleate vegetative cells, constriction and septum at the branching point of the hypha; and f. Monilioid cells.
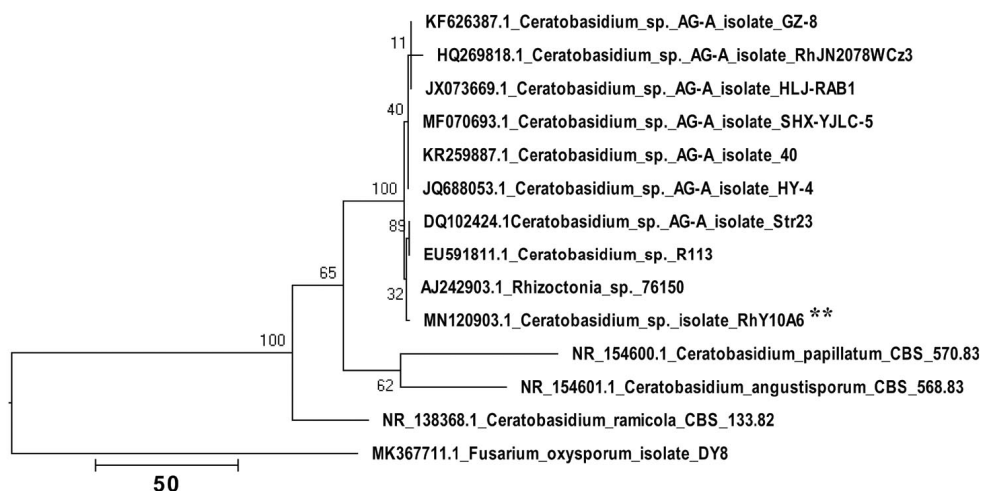

Fig. 2. Phylogenetic tree showing relationship among different isolates/species of *Ceratobasidium* based on ITS region analysis with the mycorrhizal fungus (MN120903.1) isolated from *Rhynchostylis retusa* orchid. *Fusarium oxysporium* isolate DY8 was used as an out-group. (Bar, 50 substitution per site).

The ITS region has several features that make it a strong candidate for a universal 'barcode' for fungal identification as it is easy to amplify due to high copy number, relatively few primer sets are needed as a result of the highly conserved SSU (small sub-unit) and LSU (large sub-unit) flanking regions, and varies relatively little within species but dramatically between species, and far better represented in GenBank than other loci in fungi (Taylor and McCormick 2008, Zettler and Corey 2018).

The molecular and morphological approaches established for isolation and identification mycorrhizal fungi from *R. retusa* will facilitate to elucidate the diversity and variability of the species of mycorrhizal fungi. In this way, new species of fungi could be described and the mycorrhizal fungi could be used for conservation purposes.

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