

**MORPHO-MOLECULAR CHARACTERIZATION OF *CERATOBASIDIUM* SP.:  
A MYCORRHIZAL FUNGI ISOLATED FROM A RARE EPIPHYTIC ORCHID  
*GASTROCHILUS CALCEOLARIS* (J. E. SM.) D. DON**

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**Keywords:** Orchid mycorrhiza; ITS sequencing; *Gastrochilus calceolaris*; *Ceratobasidium*.

**Abstract**

A mycorrhizal fungus, *Ceratobasidium* sp. GC (NCBI Gene Bank Accession no GQ369961) associated with the roots of an epiphytic orchid *Gastrochilus calceolaris* was investigated by cultural morphology, microscopic features and molecular analysis of Internal Transcribed Spacer (ITS) regions sequences of nuclear ribosomal DNA. The colony appearance of the fungal endophyte was fluffy growth pattern and the colour of the young colony was milky white on both surfaces that turned in to brown at maturity on the upper surface and deep brown on reverse surface. The microscopic features of the fungus *i.e.* hyphal diameter, multi-nucleate vegetative cells, right angle branching pattern with slight constriction at branching point and a dolipore septum near the branching point were observed. All the characters corroborated the identity with anamorphic *Rhizoctonia* like fungi. The ITS regions sequences of nrDNA and phylogenetic analysis based on the neighbor-joining method showed clustered with *Rhizoctonia* like fungi, and the maximum identity (98.28%) with *Ceratobasidium* RR and *Ceratobasidium* FPUB isolated from *Rhynchostylis retusa* and *Dactylorhiza hetagera*, respectively. Thus, the ITS of nrDNA sequences validated the morphological data. This is the first report of orchid mycorrhizal fungi from Bangladesh.

**Introduction**

Mycorrhizal fungi are fundamental in orchid growth and metabolism, and influence the distribution and rarity of these delicate plants. The existence of mycorrhizal associations has been established since long in orchids with certain heterogeneous groups of fungi (Hadley, 1982). It is a biotrophic mutualistic symbiosis prevalent in more than 90% of the higher plants in which both partners benefited in terms of nutrients exchange, evolution and fitness. It is one of the most significant events in the successful colonization of land by plants and the evolution of biotrophic root-inhabiting symbioses. Now-a-day, it is the most interesting area of research throughout the globe. Seed germination in orchids is totally dependent on mycobionts due to lack of sufficient food reserves (Smith and Read, 1997). Orchids are categorized as myco-heterotrophic, holomycotrophic and mixotrophic based on their nutritional dependency on fungal partner (Dearnaley, 2007). The orchids which depend on mycorrhizal fungi during seed germination stages only are referred as mycoheterotrophic. The achlorophyllous orchids entirely depend on mycorrhizal fungi for nourishment throughout their life cycle are categorized as holomycotrophic. The third orchid nutritional mode exists mixotrophy. Such orchids are photosynthetic at the adult stage but augment their nutrition requirements specially carbon *via* mycorrhizal fungi (Dearnaley, 2007). Mixotrophic orchids may be an evolutionary step between autotrophic and mycoheterotrophic orchids (Julou *et al.*, 2005). Nevertheless, all orchids are mycoheterotrophic during their early stages of growth, development and survival in nature. Mycorrhizal fungi

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colonizing roots of terrestrial orchids were well investigated for their beneficial effects on seed germination, growth and ecological fitness of the host plants (Currah *et al.*, 1987; Bidartondo, 2005). Only limited information is available for mycorrhizal fungi in epiphytic orchids which constitute a majority of orchids (Pereira *et al.*, 2003). Therefore, isolation and identification of mycobionts is of interest for understanding orchid-fungus relationship in epiphytic orchids.

From the foundation work of orchid mycorrhiza, *Rhizoctonia* spp. was considered to be the only fungal partner associated with orchids. Afterward a number of fungi identified from orchids which showed resemblance to anamorphic *Rhizoctonia* spp. in many aspects, hence are collectively called *Rhizoctonia*-like fungi (Ramsay *et al.*, 1987; Andersen and Stappers, 1994, Andersen, 1996; Shan *et al.*, 2002, Sharon *et al.*, 2008). The *Rhizoctonia* species associated with orchids include free-living saprophytes (Burgeff, 1959) and opportunistic soil pathogens. Thus, it is essential to establish the true biological entity of the mycobionts.

Conventionally the *Rhizoctonia*-like fungi are characterized based on the cultural morphology, cytomorphology of the hyphae, development of asexual resistant propagule *i.e.* monilioid cells, number of nuclei in the cells, branching pattern, nature of septum, anastomosis group (AG) etc. (Shan *et al.*, 2002). The conventional approaches for identifying orchid mycorrhizal fungi have some limitations as the majority of fungal partners are mycelia sterilia necessitating application of molecular techniques for their accurate identification (Shan *et al.*, 2002; Dearnaley, 2007; Yagame *et al.*, 2008). Fungal molecular systematics have been instrumental for the identification of orchid mycorrhizal symbionts because it overcomes the limits associated with *in vitro* isolation and morphological characterization of orchid endophytes. The aim of this study was to characterize morphologically and molecularly of the mycorrhizal fungi *Ceratobasidium* sp. GC isolated from *Gastrochilus calceolaris* and to test the coincidences between morphological and molecular characterization which will facilitate to isolate and accurate identification of mycorrhizal fungi in other orchids as well.

## Materials and Methods

### *Sample preparation and isolation of fungal endophyte*

Roots of *Gastrochilus calceolaris* (J. E. Sm.) D. Don were collected from the naturally grown plants from Bandarban district of Chittagong Hill Tracts, Bangladesh (21°11' and 22°22' N latitudes and 92°04' and 92°41'E longitudes; 300 - 500 m amsl) in June-July (rainy season) and November-December (winter season) were used for isolation of fungal symbionts. Three roots sampled from five plants were studied for estimating the percentage of cells with fungal colonization. Several transverse sections were cut at different portions of the roots attached to the tree trunk. Five thin sections taken randomly from each root were stained with lactophenol triglycero-cotton blue and observed for colonization under Labophot microscope (Nikon Corp., Japan). The incidence of fungal colonization and formation of pelotons in the root sections was calculated by following formula:

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

The root portions showing presence of fungal pelotons were surface sterilized in 4% sodium hypochlorite for 3 min, dipped in 70% ethanol for 1 min, and washed thoroughly with double sterile distilled water. The root sections 1.0–1.5 mm thick were aseptically transferred onto potato

dextrose agar (PDA) supplemented with 0.75 µg/ml streptomycin sulfate and incubated in dark at 25 °C until hyphal growth was started from explants onto the medium. The fungal endophytes those started growing from the cortex cells of the root section were considered to be putative mycorrhizal fungi. Pure cultures were obtained by successive sub-culturing of small portion of the fungal mat from the fast growing zone to fresh PDA.

#### *Study of cultural characteristics of fungal endophyte*

Colony surface and reverse colours were recorded at young and mature stages. The hyphal diameter was measured in lactophenol triglycero-cotton blue mounts on glass slides under Nikon E600 microscope (Nikon Corp., Japan). Culture growth rates were determined by inoculating uniform mycelium bits at the centre of PDA plates and measuring radial increments in colony size at 48 h interval over two weeks in three replications.

#### *Study of microscopic features of fungal endophyte*

Nuclei number in vegetative cells was determined using slightly modified method of Shan *et al.* (2002). 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 (DAPI, ProLong®, Invitrogen Ltd., Eugene, OR, UK) for 10 min, de-stained with distilled water for 2 min, and observed using 50% glycerol under Nikon E600 microscope equipped with fluorescence mode.

#### *Isolation of DNA and PCR amplification*

For extraction of DNA 1g mycelial mat from 7 days old cultures was ground under liquid nitrogen using Qiagen DNeasy Plant Kit according to the instruction provided by the manufacturer (Qiagen, CA). The amplification of ITS 1, 5.8S rRNA gene and ITS 2 was achieved using the ITS1 (5'TCCGTAGGTGAACCTGCGG) and ITS4 (5'GCTGCGTTCA TCGATGC) primers (White *et al.*, 1990). The PCR reaction was performed in 50 µl reaction mixture containing 50 ng genomic DNA, 10 pmol of each primer, 0.5 mM of dNTPs, 1× PCR buffer with 1.5 mM MgCl<sub>2</sub>, and 3 U Taq polymerase. The thermocycling conditions consisted of an initial denaturation at 94°C for 2 min, followed by 35 amplification cycles at 94°C for 1 min, 54°C for 1 min and 72°C for 2 min, and a final extension at 72°C for 8 min. The sequencing of PCR product was done by ABI Prism Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystem, CA, USA).

#### *Phylogenetic analysis*

The sequences were analyzed using the gapped BLASTn <http://www.ncbi.nlm.nih.gov> search algorithm and phylogenetic tree was constructed using the TREECON software package using Kimura's two-parameter model (Kimura, 1980) after aligning the sequences with CLUSTAL W version 1.83 (Thompson *et al.*, 1994). The ITS region sequences (~600 bp) of the fungal isolate were deposited in the NCBI GenBank database under the accession numbers GQ369961 for *Ceratobasidium* sp. strain GC (<http://www.ncbi.nlm.nih.gov/btast>).

## **Results and Discussion**

### *Fungal infestation in roots*

The fungal endophytes showed colonization in the cortex cells with peloton formation in the mature roots of the orchid (Fig. 1A). Colonization was observed only in the root portions attached with the substratum but no pelotons were found in the root tips. Colonization was absent in the aerial roots. In the colonized roots the pelotons were appeared as loose coils surrounded by live hyphae in the outer-cortex while brownish in the inner cortex. The frequency of root colonization

was significantly higher (75%) in June–July than November–December (50%) (CD = 1.02 at  $p = 0.05$ ). Seasonal variation in fungal infestation with higher colonization during the summer and rainy season of active growth and flowering as compared to the winter season of slow growth corroborated the earlier reports of higher colonization by fungi to meet the nutrient requirement for active growth and phenology of the orchids (Masuhara and Kutsuya, 1994; Siddique and Raghuvanshi, 1993; Chang, 2007).

Fungal hyphae were penetrated through root hair and passed through epidermis to the cortex and proliferated in the middle and inner layers of the cortex, forming pelotons in the cortical region of host's roots. Both young and mature pelotons were found in the root's cortex cells. Connections between the pelotons in the adjacent cortical cells through the cell wall were observed (Fig. 1A), which is a typical orchid mycorrhizal feature (Thakur *et al.*, 2018). It was also observed that hyphae did not penetrate to the endodermis and pith. Initially pelotons showed loosely coiled hyphae in the cortex cells (the 'host' phase), followed by the hyphae collapsing into the centre of the cell (the 'digestive' phase, Thakur *et al.*, 2018).

#### *Morphological characterization of fungal endophyte*

Morphological features of the fungus isolated from the roots of *G. calceolaris* showed resemblance to the *Rhizoctonia*-like orchid endophytes. The young colonies were cottony white that turned into light brown on upper surface and deep brown on reverse at maturity (Fig. 1B, C and D). A slight constriction at branching point and a dolipore septum was observed near the branching point of the new hypha (Fig. 1E). Microscopic features of the fungal endophyte are summarized in table 1. The vegetative cells of this endophyte were multinucleate (Fig. 1F) without monilioid cells were observed during the course of study even the cultures were kept for one month in growth chamber. Conventionally, *Rhizoctonia*-like fungi have been identified based on some common anamorphic features such as their cultural morphology, colony colour, mycelial characteristics *i.e.* a slight constriction at branching point of the hypha, dolipore septa, nuclear number per cell, development of monilioid cells and sclerotia (Currah *et al.*, 1987, 1988; Shan *et al.*, 2002; Pereira *et al.*, 2003; Zhu *et al.*, 2008). The characteristics of the fungal endophyte isolated from *G. calceolaris* was, therefore, corroborated the identity with *Rhizoctonia*-like fungi belonging to the genus *Ceratobasidium*. *Rhizoctonia*-like fungi represent an assemblage of taxonomically diverse groups that differ in cultural and morphological features, including anamorph and teleomorph stages (Currah *et al.*, 1987). The formation of asexual resistant propagules (monilioid cells), dolipore septum, constriction at branching point of the hypha, bi- or multinucleate vegetative and monilioid cells are the common anamorphic features of *Rhizoctonia*-like fungi widely reported as orchid mycorrhizal fungi (Currah *et al.*, 1997; Sneh *et al.*, 1991; Otero *et al.*, 2002; Shan *et al.*, 2002). Nuclear number per cell varies from uni-, bi- to multinucleate in different orchid mycorrhizal fungi and even in different strains of the same species (Sneh *et al.*, 1991; Otero *et al.*, 2002; Pereira *et al.*, 2014). This framework adopted by several researchers for identifying orchid mycorrhizal fungi offers a taxonomically correct and justified approach to define taxa (Shan *et al.*, 2002). Sexual structures are generally more informative for taxonomy and systematics than the vegetative structures. However, fungi placed under the form-genus *Rhizoctonia* seldom reveal their basidiocarps, hence often referred to and identified by their anamorphs. *Rhizoctonia*-like fungi represent the genera *Ceratorhiza*, *Epulorhiza*, *Monilioopsis*, *Rhizoctonia*, *Ceratobasidium*, *Thanatephorus*, *Tulasnella*, and *Sebacina* (Warcup and Talbot, 1966). Species concepts within these genera can be corroborated based on finer culture characteristics such as mycelial morphology on specific media (Currah *et al.*, 1990; Zelmer and Currah 1995; Andersen, 1996).

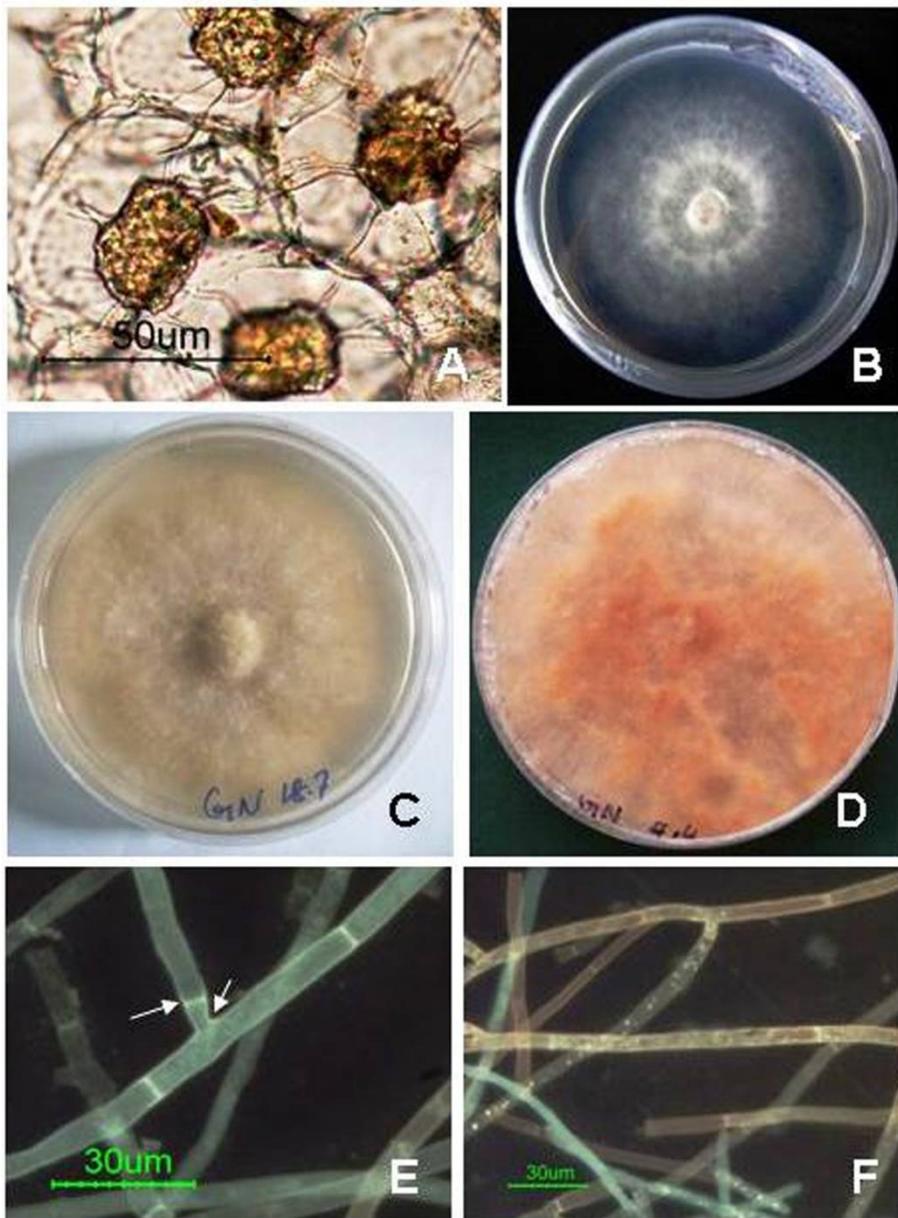


Fig. 1. A) Fungal pelotons in the T.S. of root of *G. calceolaris*, B) Five days old young colony of fungal endophyte on PDA surface, C-D) Fifteen days old mature colony front view and reverse view respectively, E) Fungal hyphae showing constriction and dolipore septum near the branching point (arrow), and F) Showing multinucleate vegetative cells after DAPI staining.

#### *Phylogenetic analysis*

The BLASTn search of ITS region sequences of nrDNA of the fungal isolates from *G. calceolaris* showed maximum identity with *Ceratobasidium* spp. reported as the widespread fungi developing mycorrhizal association with orchids (Otero *et al.*, 2002). The phylogenetic tree was

constructed based on the ITS region sequences of the fungal isolates and their closely related species formed distinct taxonomic group (Fig. 2). The ITS region sequences of *Ceratobasidium* sp. GC showed 98.28% similarity with *Ceratobasidium* sp. RR and 98.27% similarity with *Ceratobasidium* sp. FPUB 168, *Rhizoctonia* sp. M2ao1, *Rhizoctonia* sp. Abn1b and *Rhizoctonia* sp. Onv6. These fungi were isolated from *Rhynchostylis retusa*, *Dactylorhiza hatagirea*, *Aerides oradaia*, *Aranda Brite Ng*, *Oncidium varimyce* × *Oncidium nona*, respectively (Ma *et al.*, 2001; Otero *et al.*, 2004; Aggarwal *et al.*, 2007, Hossain *et al.*, 2013). The ITS of nrDNA sequences data validated the morphological data and reconfirm the identity of the fungal endophyte isolated from *G. calceolaris* as *Rhizoctonia*-like fungi, *Ceratobasidium* sp.

**Table 1. Morphological characteristics of *Ceratobasidium* sp. str. GC isolated from *Gastrochilus calceolaris*.**

Parameter	Characteristics
Colour of young colony (surface)	Cottony white
Colour of young colony (reverse)	Brownish white
Colour of mature colony (surface)	Light brown
Colour of mature colony (reverse)	Deep brown
Colony appearance	fluffy growth pattern
Colour of vegetative hyphae	Hyaline
Diameter of vegetative hyphae	8-12 µm
Shape of monilioid cells	Not found
Dimension of monilioid cells	-
Branching pattern	Right angle with slight constriction at the branching point
Colony growth rate (mm/hr)	0.033-0.045
Nature of septum	Dolipore septum
Spore	Absent
Nuclear condition in vegetative cell	Multinucleate

The conventional approaches for identifying orchid mycorrhizal fungi have limitations as most of the fungal endophytes are mycelia sterilia. Consequently, the broad vegetative criteria for identification have resulted in paraphyletic taxonomy with various unrelated fungi being grouped together necessitating the application of molecular techniques for accurate identification (Otero *et al.*, 2002; Shan *et al.*, 2002; Dearnaley, 2007; Yagame *et al.*, 2008). ITS region sequencing is the common and powerful molecular technique for accurate identification of orchid mycorrhizal fungi (Kuninaga *et al.*, 1997; Taylor and Bruns, 1999; Salazar *et al.*, 2000; Gonzalez *et al.*, 2001; Pope and Carter, 2001; Sharon *et al.*, 2008). 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 and 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 ITS region characterization of the endophytes isolated from *G. calceolaris* confirmed the identity with *Ceratobasidium* spp.

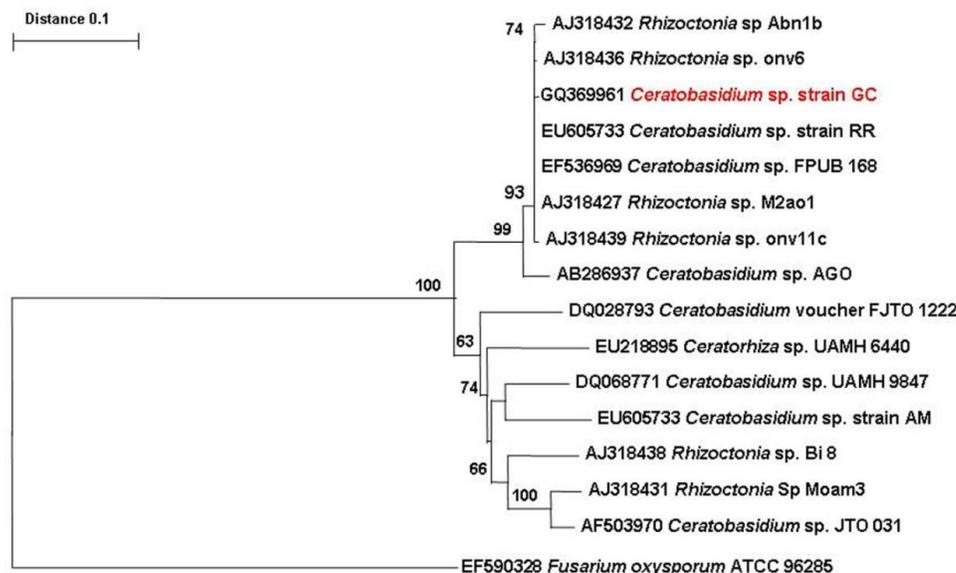


Fig. 2. Phylogenetic tree showing relationship among *Ceratobasidium* sp. strain GC and representatives of some related taxa based on ITS region analysis. Numbers on the nodes indicated bootstrap values. *Fusarium oxysporum* ATCC 96285 was used as the out-group. Bar, 0.1 substitution per site.

### Conclusion

The morpho-molecular identification technique established for the root endophytic fungi *Ceratobasidium* sp. from *G. calceolaris* will help in easy and accurate identification of other orchid mycorrhizal fungi as well. This is also the first report on isolation, characterization and phylogenetic analysis of orchid mycorrhiza from Bangladesh.

**Conflict of interest:** The author has no conflict of interest.

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(Manuscript received on 10 May, 2019; revised on 10 December, 2019)