MULTILOCUS GENE PHYLOGENY REVEALS OCCURRENCE OF COLLETOTRICHUM CYMIDICOLA AND C. CLIVIAE ON ORCHIDS IN NORTH EAST INDIA

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SUMMARY

The causal organism responsible for severe outbreaks of orchid anthracnose in India, which affects leaves, petioles and blooms, is not clearly established. Ten Colletotrichum isolates recovered from different orchid species in the Sikkim state were characterized based on morphological and a multilocus molecular phylogenetic analysis of the rDNA-ITS region (ITS), partial actin (ACT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequences. Phylogeny inferred from combined datasets of ACT, ITS and GAPDH revealed two groups, representing Colletotrichum cymbidiicola and C. cliviae. Cultural and morphological characters are presented for these two species. Pathogenicity assays confirmed that both species isolated from orchids are the causal agents of anthracnose. The occurrence of C. cymbidiicola and C. cliviae is reported for the first time on orchids in India.

Key words: orchids, anthracnose, Colletotrichum, phylogeny, ITS, ACT, GAPDH

INTRODUCTION

Orchids are highly valued for cut-flower production and as potted plants (Huang et al., 2009), account for about 10% of the global floriculture trade and constitute a multi billion dollar industry (Medhi and Chakrabarti, 2009). About 1,300 species of orchids are native to India and some 800 are found in the North Eastern region of the country, which is the centre of origin for important species such as Cymbidium. Currently, many of the orchid genera are threatened or might have already disappeared in the wild. In recent years, commercial cultivation of orchids for export has started in the north-eastern hill and coastal regions of Karnataka and Kerala states (Medhi and Chakrabarti, 2009).

The genus Colletotrichum is considered the eighth most important group of plant pathogenic fungi (Dean et al., 2012). The genus occurs predominantly in tropical and subtropical regions on a wide range of crops, and is primarily described as causing anthracnose diseases (Cannon et al., 2012). Anthracnose caused by Colletotrichum species has been reported to cause huge losses to field- and nursery-grown orchids (Duff and Daly, 2002). Studies on the causal agents of anthracnose disease of orchids are few. Recent reports have identified Colletotrichum cymbidiicola on Cymbidium, C. oncidii on Oncidium, and an unidentiﬁed species of the C. boninense complex on Dendrobium (Damm et al., 2012), C. gloeosporioides on Cymbidium sinense in China (Huang et al., 2012) and C. karstii on Phalaenopsis in USA (Jadrane et al., 2012). In India, anthracnose caused by Glomerella cingulata was ﬁrst reported on Vanda coerulia (Ramakrisnan et al., 1952). Later C. orbiculare was reported as the causal agent of anthracnose on Phalaenopsis hybrids, Cymbidium aloifolium, Dendrobium nobile, Bulbophyllum clyndrum and Coelogyne flaccida from Uttarakhand and Assam (Roy, 1979; Roy and Barman, 1979; Sohi, 1992). In Maharashtra, the agent of anthracnose on Dendrobium latifolium and Phalaenopsis sp. was identified as Colletotrichum dendrobl (Yadav, 1980). Whereas severe outbreaks of anthracnose on Oncidium sp. from Kerala were attributed to Glomerella cingulata (Shreedharan et al., 1994). Srivastava (1999) identiﬁed G. cingulata as the cause of anthracnose in Sikkim. A recent survey in Sikkim identiﬁed severe anthracnose on 85 species of 29 orchid genera (Bag, 2007) but the pathogen’s species was not described except for a single isolate which was recently identiﬁed as C. cymbidiicola (Sharma and Shenoy, 2013). It is not clear, however, whether the anthracnose of all orchids in India is due to C. cymbidiicola or if other species that have been isolated from orchids are equally pathogenic.

There are limitations to the traditional identiﬁcation of Colletotrichum species based on morphology, due to the plasticity of morphological traits (Sutton, 1992). A Colletotrichum database is already available at http://www.cbs.knaw.nl/Colletotrichum/ for species identiﬁcation based on morphology and multi-locus gene phylogenies. To our knowledge, no systemic research work has been undertaken to determine which Colletotrichum species are
responsible for orchid anthracnose in Sikkim, which is a potential area for orchid cultivation. Thus, the objective of the present study was to determine and characterise the species of *Colletotrichum* associated with anthracnose of orchids in India, based on morphology and multi-locus gene phylogeny.

**MATERIALS AND METHODS**

**Isolation of *Colletotrichum* species from orchids.** *Colletotrichum* was isolated from leaves of different orchids (Table 1) showing typical anthracnose symptoms collected at the National Research Centre for Orchids at Pakyong (Sikkim) during July-October of 2010 and 2011. Three types of symptoms were observed, i.e. necrosis of leaf tips proceeding towards the base of the blade, scattered black sunken spots, dark brown or light gray patches with concentric rings (Fig. 1). Fungal isolates were recovered from leaf lesions by removing three 5 × 5 mm pieces of tissue from the edge of the infected area. These were surface-sterilized by dipping in 1% sodium hypochlorite for 1 min, then in 70% ethanol for 1 min, rinsed three times with sterilized water and dried on sterilized tissue paper (Cai et al., 2009), then plated in potato dextrose agar (PDA) amended with streptomycin (100 µg/ml) and incubated at 25±1°C in the dark. Single-spore cultures were obtained from each *Colletotrichum* isolate according to Goh (1999). Pure cultures were maintained on PDA slants at 10°C by sub-culturing at 4-week intervals. Three agar plugs (3 mm diam) from actively growing cultures on PDA were suspended in 3 ml of 20% glycerol: 17% skimmed milk (1:1) solution and placed at −80°C (Chowdappa et al., 2009) for long term storage.

**Morphological traits.** Cultures for morphological analyses were grown in 90 mm Petri plates containing 15 ml PDA under constant fluorescent light at 25±1°C for 7 days. Colony diameter was measured daily for 7 days and growth rate was calculated as the 7-day average of the mean daily radial growth for each culture, and expressed as mm/day. After seven days, colony size, colour of the conidial masses and zonation were recorded. Colony colours were scored according to Rayner (1970). Conidial shape was recorded in sterile distilled water for conidia harvested directly from cultures. Three replicates for each isolate were maintained and the experiment was repeated three times. Each replicate contained three plates. Appressoria were produced in slide culture (Johnston and Jones, 1997). About 3 cm squares of PDA were deposited on a glass slide and placed in an empty Petri dish containing filter paper moistened with sterile distilled water. The edge of the agar was inoculated with spores on all four sides and a sterile cover slip was placed over the inoculated agar. The Petri plates were sealed with parafilm and incubated at 25±1°C on a laboratory bench. Appressoria were formed after 4-7 days across the underside of the cover slip. Cover slips were placed onto a drop of lactophenol blue stain on fresh microscope slides. Length and width of 100 randomly chosen conidia and appressoria were determined for each isolate at 400× magnification under Zeiss bright field microscope using an Axio Vision software.

**DNA extraction.** Cultures were grown in 100 ml of potato dextrose broth in 250 ml conical flasks for 7 days at 25±1°C. Mycelium was harvested from liquid cultures by filtration through Whatman No.3 filter paper, damp dried and subsequently ground into a fine powder in liquid nitrogen. DNA was extracted from the frozen mycelial powder by the method of Raeder and Broda (1985) slightly modified by Chowdappa et al. (2003) by incubating at 37°C for 10 min after the phenol:chloroform:isoamylalcohol (25:24:1) precipitation. This was followed by precipitation with 0.54 vol. of isopropyl alcohol and centrifugation at 10,000 rpm for 2 min. The DNA pellet was washed with 70% cold ethanol, dried at room temperature overnight (16 h), then resuspended in 30 µl 10 mM TE buffer (pH 8). DNA was stored at −20°C.

**Multilocus genotyping.** The complete rDNA-ITS (ITS) region, partial actin (ACT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were employed for multilocus genotyping as sequences of these three genes are only available for all 107 type species at *Colletotrichum* database (http://cbs.knaw.nl/colletotrichum). The complete rDNA-ITS region was amplified with primer pair ITS1 (5’TCCGTAGGTGAAACTTGGG-3’) and ITS4 (5’T CCTCCGCTTTATTGATATGC-3’) (White et al., 1990). PCR reactions (50 µl) were performed in an Eppendorf master cycler by 34 cycles of denaturation at 94°C for 60 sec, annealing at 55°C for 60 sec, and extension at 72°C for 1.5 min with an initial denaturation of 4 min at 94°C before cycling and final extension of 5 min at 72°C after cycling. Part of the actin gene was amplified using primer pair ACT512F (5’-ATGTGCAAGGCGGGTTTGC-3’)

![Fig. 1. Anthracnose symptoms: a. Cymbidium hybrid; b, Coelogyne elata; c, Dendrobium pendulum.](image-url)
and ACT783R (5’-TACGAGTCTCTTGGCAGGAT3’) (Prihastuti et al., 2009). The cycling parameters consisted of a 3 min initial denaturing step at 95°C followed by 34 cycles denaturation at 95°C for 1 min, annealing at 52°C for 30 sec, extension at 72°C for 1 min and a final extension of 10 min at 72°C after cycling. The second intron of the glyceraldehyde 3-phosphate dehydrogenase gene was amplified with the primer pair GDF1 (5’-GCCGTCAACGCCCTTACTGTA3’) and GDR1 (5’-GGGTGAGTCGTACTTGACATGT3’) (Guerber et al., 2003). The cycling parameters consisted of an initial denaturation step at 94°C for 4 min, followed by 34 cycles of denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, extension at 72°C for 1 min and a final extension at 72°C for 10 min after cycling.

PCR amplifications were confirmed by observing products in 1% agarose electrophoresis gels stained with ethidium bromide. Amplified DNA products for the ITS, ACT and GAPDH were purified using a Nucleospin extract II. Products were custom sequenced in both directions (Bioserve Technologies, India).

**Phylogenetic analyses.** The dataset for ITS, ACT and GAPDH gene regions for the Colletotrichum isolates were combined after ILD test along with the reference sequences of 107 type species from the Fungal Biodiversity Centre, Utrecht, the Netherlands (CBS-KNAW) (http://www.cbs.knaw.nl/Colletotrichum/). The sequences were aligned with Clustal W, Bioedit v.7.0.5.3 (Hall, 1999) and the alignment gaps were treated as missing data. Model Test v.0.1.1 (Posada, 2008) was used to carry out the corrected Akaike information criteria (AICc) for statistical selection of best-fit models of nucleotide substitution. MrBayes v3.1.2 was used to generate phylogenetic trees with a Markov Chain Monte Carlo (MCMC) algorithm with Bayesian probabilities used for the combined sequence datasets which provides the measurement of clade support as posterior probabilities (Ronquist and Huelsenbeck, 2003). MCMC were run for 20 million generations and for every 10000 generations sampling was done with the first 50% of samples discarded as burn-in. Maximum likelihood (ML) analyses was performed in RAxML v7.0.4 (Stamatakis, 2006), with the thorough bootstrap algorithm of RAxML with 1000 replications implemented partitioning by gene with nodal support in act. For the resulting tree, consistency index (CI) and retention index (RI) were calculated employing PAUP 4.0b10 (Swofford, 2003). The robustness of the trees was measured by 500 bootstrap replicates and addition of 10 random sequences (Felsenstein, 1985).

**Pathogenicity.** Pathogenicity of Colletotrichum isolates was determined on mature detached leaves obtained from one-year-old Cymbidium hybrid, Erria bambusofolia, Erria amica, Dendrobium fimbriatum, Bulbophyllum hirtum, Oncidium spinacealatum, Coleogyne clala and Cymbidium pendulum plants grown at 25 ±1°C in a greenhouse with a 12 h photoperiod. The pathogenicity assays of fungal isolates were performed on orchid hosts from where they were isolated. Fungal isolates were grown on potato dextrose agar (PDA) plates for seven days under cool white fluorescent light (67.5 mmol m−2 s−1) at 25 ±1°C to promote sporulation. Conidia were washed from PDA plates with 5 ml of sterile distilled water and adjusted to 1.5x10⁶ conidia/ml using a haemocytometer (Than et al., 2008). Leaves were disinfected with 1% sodium hypochlorite for two min, and washed three times with distilled water. The leaves were blotted dry with a sterilized tissue paper and inoculated by using wound/drop inoculation method (Lin et al., 2002; Than et al., 2008). Control leaves were inoculated with 10 µl of sterilized distilled water onto the wound. Inoculated leaves were incubated at 25 ±1°C with a 12 h photoperiod for seven days in plastic Petri dishes (90 mm) that contained a water-soaked cotton wad to maintain humidity. Three replicates were used for each isolate and the experiment was repeated three times. Each replicate contained six leaves. The diameter of the anthracnose lesions developed on the leaves was measured seven days post inoculation.

**RESULTS**

**Collection of isolates.** Ten isolates of Colletotrichum spp. were recovered from infected orchids showing typical symptoms of anthracnose (Table 1). Of the 10 isolates, nine were identified as C. cymbidiicola and one isolate as C. cliviae, based on morphology and multilocus gene phylogeny.

**Morphological comparisons.** The cultures of C. cymbidiicola had cottony white aerial mycelium on the upper surface and olivaceous grey in the lower surface (Fig. 2). The colonies of C. cliviae were olive grey on the upper surface and greenish black in the lower surface. The growth rate of C. cymbidiicola isolates ranged from 3.9 to 4.5 mm day⁻¹ whereas C. cliviae exhibited higher growth rate of 15.9 mm day⁻¹. Conidia of both fungal species were hyaline, smooth-walled, single-celled and straight. Conidia of C. cymbidiicola isolates were cylindrical with rounded ends and a hilum-like protuberance at the base, and contained two or three large guttules (Fig. 2). Isolates of C. cliviae had cylindrical, slightly curved conidia with obtuse ends. The size of conidia of C. cymbidiicola isolates varied from 13.9-15.8 × 5.6-5.9 μm with L/B ratio from 2.48 to 2.81. Isolate of C. cliviae had conidial sizes of 17.2 × 5.5 μm with L/B ratio of 3.18 (Table 2). Appressoria of C. cymbidiicola had lobate margins and measured 11.23-12.67 × 6.02-6.75 μm, whereas those of C. cliviae were irregular and lobed with sizes of 10.47 x7.02 μm (Table 2 and Fig. 2)

**Phylogenetic analyses.** In phylogenetic analyses, DNA sequences of the three genes derived from all 10 isolates
were concatenated to form a matrix of 1122. The gene boundaries in the alignment were ITS: 1-563, ACT: 564-838, GAPDH: 839-1122. The analysis involved 117 nucleotide sequences including the outgroup Monilochaetes infuscans CBS 869.96 (Fig. 3). For Bayesian analysis, a HKY+I model was selected for ITS, a GTR+G model for ACT and a HKY+G model for GAPDH was incorporated in the analysis. The consensus tree obtained from Bayesian analyses confirmed that the tree topology obtained with the three different models was similar. Datasets were combined on the basis of ILD test, with the final models as GTR+I+G to obtain the final consensus tree. Bayesian posterior probability values agreed with bootstrap supports. Tree showed CI=0.331 and RI=0.793 for all sites generated during the MP analysis. The dataset of combined three genes comprised 1122 characters, 718 of which were parsimony informative. The isolates of Colletotrichum associated with anthracnose of orchids clustered into two well defined groups, representing two distinct species. Group I contained OORC 7 and clustered along with C. cliviae (CBS 125375). Isolates OORC1, OORC3, OORC10, OORC13, OORC16, OORC18, OORC19, OORC23 and OORC24 constituted a separate lineage and appeared similar to C. cymbidiicola (CBS 123757) in group 2. C. cymbidiicola was recovered from Cymbidium hybrid (OORC1), Eria bambusifolia (OORC3), Eria amica (OORC10), Cymbidium hybrid (OORC13), Dendrobium fimbriatum (OORC16), Bulbophyllum birtum (OORC18), Oncidium spacementum (OORC19), Coelogyne clala (OORC23) and Liparis longipes (OORC24) and C. cliviae on Cymbidium pendulum (OORC7).

Pathogenicity. When wound-inoculated, C. cymbidiicola, and C. cliviae produced typical symptoms of anthracnose on the leaves of respective orchid host from where they had been recovered (Table 2). All of the isolates were

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**Table 1. Colletotrichum isolates used in this study.**

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Colletotrichum species</th>
<th>Host</th>
<th>Locality</th>
<th>Actin</th>
<th>GADPH</th>
<th>ITS</th>
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<td>C. cymbidiicola</td>
<td>Cymbidium hybrid</td>
<td>Pakyong</td>
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<td>JX902353</td>
<td>JX902417</td>
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<td>Eria bambusifolia</td>
<td>Pakyong</td>
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<td>Pakyong</td>
<td>JX902320</td>
<td>JX902358</td>
<td>JX902421</td>
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<tr>
<td>OORC13</td>
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<td>Cymbidium hybrid</td>
<td>Pakyong</td>
<td>JX902331</td>
<td>JX902359</td>
<td>JX902419</td>
</tr>
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<td>Dendrobium fimbriatum</td>
<td>Pakyong</td>
<td>JX902332</td>
<td>JX902361</td>
<td>JX902423</td>
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<td>OORC18</td>
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<td>Bulbophyllum birtum</td>
<td>Pakyong</td>
<td>JX902321</td>
<td>JX902362</td>
<td>JX902424</td>
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<td>Oncidium spacementum</td>
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<td>Liparis longipes</td>
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<td>JX902369</td>
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<td>Cymbidium pendulum</td>
<td>Pakyong</td>
<td>JX902334</td>
<td>JX902356</td>
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</tr>
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</table>

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**Fig. 2.** Morphological traits of Colletotrichum cliviae (1) and Colletotrichum cymbidiicola (2). a, culture (upper surface); b, culture (reverse); c, conidia; d, appressoria.
re-isolated from inoculated leaves and showed the same morphological characteristics that were observed upon the initial isolation.

**DISCUSSION**

The accurate identification of *Colletotrichum* species associated with orchids is important for implementing effective control strategies. Cai et al. (2009) reviewed the taxonomic approaches for studying *Colletotrichum* species and recommended a polyphasic approach to discriminate *Colletotrichum* species, as single phenotypic approaches proved to be inadequate. In the current study, we identified isolates belonging to *C. cymbidiicola* and *C. cliviae* as responsible for anthracnose disease of orchids in India based on a combined application of morphological characters and multi-loci phylogeny. This is the first report of occurrence of *C. cymbidiicola* and *C. cliviae* on orchids in India.

Recent multilocus phylogenetic studies (Cannon et al., 2012; Damm et al., 2012; Weir et al., 2012) have transformed the taxonomic status of *Colletotrichum* species. Damm et al., (2012) identified *C. cymbidiicola* as one of the 17 species of the *C. boninense* species complex. *C. cymbidiicola*, which was named after the host plant, is a presumably host-specific plant pathogen, reported from *Cymbidium* sp. only (Damm et al., 2012). In this study, *C. cymbidiicola* was encountered on *Cymbidium* hybrids, *Eria bambusifolia*, *Eria amica*, *Dendrobium fimbriatum*, *Bulbophyllum bambosifolia*, *Oncidium spacealatum*, *Coelogyne clava*, and *Liparis longipes*, indicating that it is not specific to *Cymbidium* sp. *C. cliviae*, first isolated from *Clivia miniata* in China, was reported as not being host-specific (Yang et al., 2009), an instance confirmed in the present study by its recovery from *Cymbidium pendulum*. Pathogenicity tests showed that the *Colletotrichum* species isolated from infected orchid leaves are pathogenic to the respective hosts, hence they can be regarded as the casual agents of orchid anthracnose.

To our knowledge, this is the first systematic study of *Colletotrichum* species on orchids in India and the first record of *C. cymbidiicola* and *C. cliviae* in this country. The results generated in this study enhance our understanding of the orchid/Colletotrichum pathosystem and provide basic information for implementing plant quarantine
Fig. 3. Phylogenetic tree constructed with concatenated sequences of the ITS, ACT and GAPDH genes. Bayesian posterior probability values above 0.50 are shown at the nodes and bootstrap support values (500 replicates) above 50% below BPP values.
strategies and for developing effective disease management strategies including the development of resistant cultivars for use in commercial orchid production.

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