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SHORT COMMUNICATION

Additions to *Gliocephalotrichum* species (anamorphic Hypocreales) from fruit litter of the medicinal plant *Terminalia chebula* in the Western Ghats, India

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Abstract Two species of *Gliocephalotrichum* were isolated from fallen fruits of the medicinal plant *Terminalia chebula* collected from the forests of Western Ghats, India. On the basis of morphological characters and ITS1–5.8S–ITS2 sequence similarity, the fungi have been identified as *Gliocephalotrichum longibrachium* and *G. bulbilium*. For the former species, this is the first report of its occurrence from India, whereas the latter, showing significant morphological variability, has been known previously from India.

Keywords Anamorphic fungi · ITS sequence · Morphology · New record

The Western Ghats comprise mountain ranges in southwestern India that are rich in evergreen rainforests. They are recognized as biodiversity hotspots by virtue of their rich flora and fauna, including endemic species. Our studies on fungal diversity have led to the identification of several novel fungi from this region (Singh et al. 2009, 2010; Senthilarasu et al. 2010; Karandikar and Singh 2010; Rajeshkumar et al. 2010). *Terminalia chebula* L. (Combretaceae), a medicinal tree growing extensively in the region, is widely used in ayurvedic medicines in India for ailments such as wounds, ulcer, inflammation, skin diseases, jaundice, and renal calculi. From decaying fruits associated with the moist litter (fruit litter), two fungi referable to the genus *Gliocephalotrichum* Ellis & Hesseltine (1962) were isolated and investigated for their taxonomic identity and in vitro cultural characteristics. Identification of the two fungi as *Gliocephalotrichum bulbilium* J.J. Ellis & C.W. Hesseltine and *Gliocephalotrichum longibrachium* Decock & S. Huret has been based on morphological characteristics of the sporulation as well as sequencing of internal transcribed spacer (ITS) regions of the rDNA. One species, *G. longibrachium* Decock & S. Huret, is a first report for the country (Bilgrami et al. 1991; Jamaluddin et al. 2004), whereas *G. bulbilium* J.J. Ellis & C.W. Hesseltine is redocumented with taxonomic details recorded from in vitro culture lacking in previous reports (Jamaluddin et al. 1972; Arya 1994). The present study describes the two *Gliocephalotrichum* species and reveals their intraspecific morphological and molecular variation.

The decaying fruits of *T. chebula* (Combretaceae) collected from Mahabaleshwar forests (17°55′15″N, 73°39′21″E) of Maharashtra, India, in October 2008 were given a mild surface sterilization with 70% ethanol followed by incubation in a moist chamber at 25°C. From the cultures that developed, spores were picked up and streaked on agar plates, and spore suspensions in sterile water were plated out using the dilution poured plate technique. Potato dextrose agar (PDA), V-8 juice agar (V8), cornmeal agar (CMA), and malt extract agar (MEA) supported good growth and sporulation, and cultural characters of the fungi were studied on these media after incubation at 25°C. Specimens mounted in lactophenol-cotton blue were photographed with an Olympus

The herbarium sample is deposited in Ajrekar Mycological Herbarium (AMH 9279) at MACS' Agharkar Research Institute, Pune, India (AMH, according to Holmgren et al. 1990), and the in vitro pure cultures isolated from designated herbarium specimen are deposited in National Fungal Culture Collection of India (NFCCI-WDCM 932), MACS' Agharkar Research Institute, Pune, India.

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CX-41 light microscope. The sizes of different fruiting structures recorded are the extreme values of 50 measurements, and in some cases an average of these measurements is shown in parentheses.

The specimen (dried fruit) has been deposited in Ajrekar Mycological Herbarium (AMH, according to Holmgren et al. 1990) with the accession no. AMH 9279; in vitro pure culture isolated from *T. chebula* fruits has been deposited in National Fungal Culture Collection of India (NFCCI-WDCM 932) with the accession numbers NFCCI 1494 (*G. bulbilium*) and NFCCI 1921 (*G. longibrachium*), MACS' Agharkar Research Institute, Pune, India.

Mycelial culture was homogenized in a FastPrep 24 tissue homogenizer (MP Biomedicals, Germany) followed by nuclear DNA extraction by the CTAB method of Gräser et al. (1999). The polymerase chain reaction (PCR) amplification of the ITS region (ITS1–5.8S–ITS2) and subsequent sequencing was performed as described earlier (Singh et al. 2010). Ribosomal DNA sequences have been deposited in GenBank with the accession numbers HQ171605 (*G. bulbilium*) and HQ171606 (*G. longibrachium*).

For phylogenetic study, related sequences (including all known *Gliocephalitrichum* species) were retrieved in FASTA format from GenBank. Sequence alignment of ITS region of 30 strains was done using CLUSTAL W and analyzed by the neighbor-joining method using the Kimura two-parameter model by MEGA ver. 5 (Tamura et al. 2011). ITS sequences of *Gliocephalotrichum* species isolated from India were also aligned manually with the authentic sequences of the types of all known species of the genus for identifying variable loci. The sequence alignment has been submitted in TreeBASE database.

Comparative analysis of morphological features when cultivated on different media such as PDA, CMA, V-8 juice agar, and MEA showed wide variation in number and position of the sterile arms, conidiophore length, and number and position of the penicillus, particularly in the isolate referable to *G. bulbilium* (Nicot 1967; Decock et al. 2006; Singh et al. 2010). The technical descriptions and the taxonomic identity of the two isolates are as follows.

Gliocephalotrichum longibrachium C. Decock et S. Huret. Mycologia 98:489, 2006 Fig. 1a–c

Colonies on PDA 75–80 mm in diameter in 5 days, floccose, center pale mouse grey, and margin olivaceous buff with abundant aerial mycelium and conidial masses. Chlamydospores abundant. On V8, 80–85 mm in diameter in 5 days, hyaline, later turning to yellow with abundant sporulation. On CMA, 80–85 mm diameter in 5 days, floccose, raised, whitish, aerial mycelium turns yellowish to olivaceous with sporulation.

Emerging hyphae branched, hyaline to pale yellow, 5–11 µm wide. Conidiophores mononematous, macronematous, arising directly from submerged hyphae and from chlamydospores, hyaline to subhyaline,1–2-septate (rarely 1–3-septate), 140–225 µm long (avg. 203.3 µm), 7.5–15 µm wide at the base (avg. 11.56 µm), 6.5–11 µm wide at the apex, base bulbous and swollen, conidiophores bearing a compact penicillate head and sterile arms at the apex; penicillus with successive branches; primary 3–6, cylindrical, thick walled, 10–20 × 3–6.5 µm; secondary 3–4, cylindrical to slightly clavate, 6–10 × 3–5 µm; tertiary (3–4), 5–8 × 2.5–4 µm, bearing 2–3 quaternary branches or 3–6 phialides with conidia. Conidia hyaline, cylindrical to fuisoid, base rounded, without globules, 5–8.5 × 1–2.0 µm. Sterile arms 2–7, subtending to the main penicillus, arising at right angle from conidiophores, growing upward above the conidial heads, hyaline, septate, thick walled, apical cell spatulate to clavate, 50–250 µm long, 3.5–7.5 µm wide at the base, 2.5–3.5 µm at the apex. Chlamydospores rounded in long chain, or in irregularly shaped clusters, thick walled, light brown to brown, individual cells 6–28 × 8–30 µm.

Material examined: India, Maharashtra, Mahabaleshwar; on decomposing fruits of *Terminalia chebula*, August 2009, L.S. Yadav, in vitro culture no. NFCCI 1921.

Gliocephalotrichum bulbilium Ellis J.J. et C.W. Hesseltine. Bull. Torrey Bot. Club 89: 22, 1962 Fig. 1d–f

Colonies on PDA 80 mm in 3 days at 25°C, floccose, center buff, margin dull white to buff, reverse dull white, sporulating well with chlamydospores at center. Conidiophores mononematous, macronematous, arising from submerged hyphae, hyaline to subhyaline, minutely roughened, 4–12 septate, 187–575 × 7.5–15 µm (avg. 332 × 10.6), bearing a compact penicillus-like head. Penicillus hyaline with 3–5 successive branches; primary branches 15–35 × 7.5–12.5 µm (avg. 27.9 × 8.08), secondary branches (2–4), 12.5–15 × 5–12.5 µm (avg. 13.3 × 6.1); tertiary branches (2–3), 7.5–8.0 × 4.5–5.0 µm (avg. 7.7 × 4.6). Conidia variable in shape**,** oblong to cylindrical or allantoid, straight to curved, sometimes with curved tip, hyaline, 6–12.5 × 2–2.5 µm (avg. 9.8 × 2.2 µm). Sterile arms (2–9) directly subtending to the penicillus, arising at right angle from conidiophores, sometimes arising below at irregular distance from penicillus and from two different positions at conidiophores, hyaline, thick walled, 5–12 septate, 350–550 µm long (avg. 418), base 5–7.5 µm wide, middle 2.5–3.5 µm, and apex 1–2 µm. Chlamydospores intercalary, muriform, globose to elongated, thick walled, light, normal to golden brown, 50–70 × 50–85 µm diameter (avg. 50 × 69.2).

Material examined: India, Maharashtra, Mahabaleshwar; on decomposing fruits of *Terminalia chebula*, Oct.

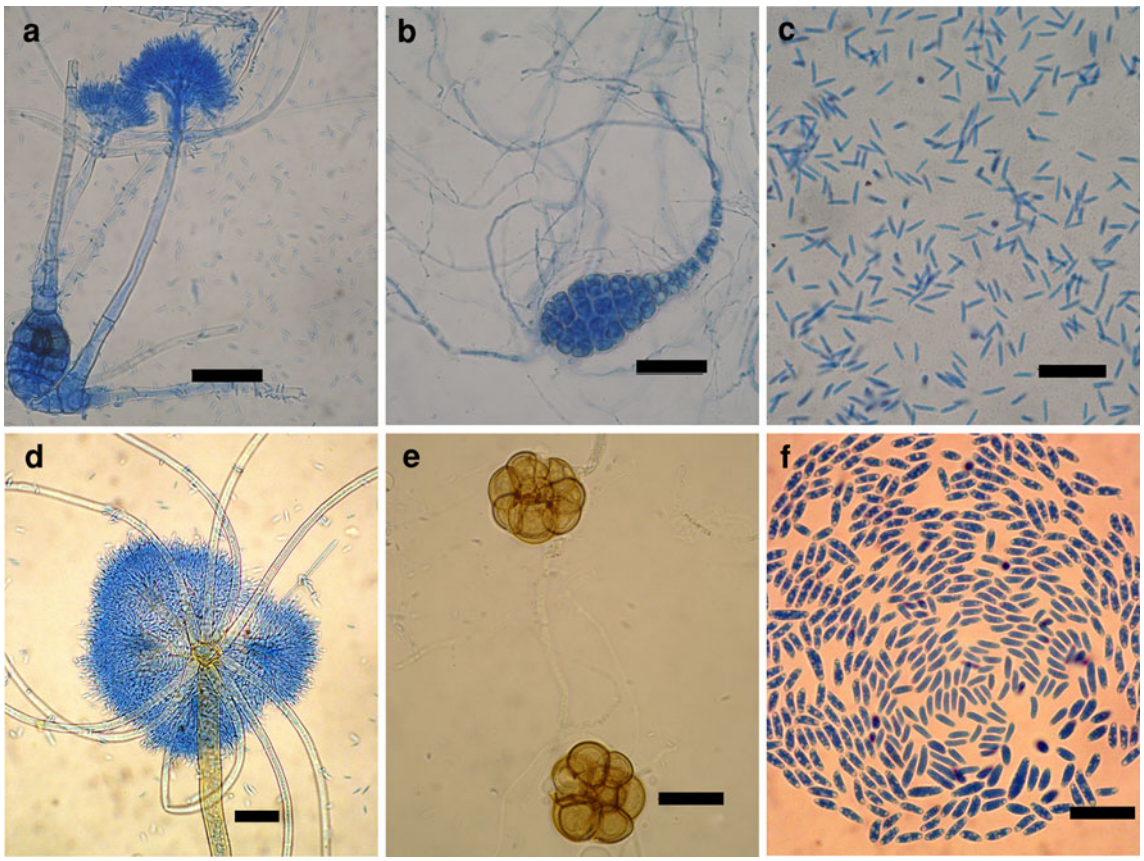

Fig. 1 *Gliocephalotrichum longibrachium*: habit showing conidiophores with sterile arms (**a**); chlamydospore (**b**); conidia (**c**). *Gliocephalotrichum bulbilium*: habit showing penicillus and sterile arms (**d**); chlamydospores (**e**); conidia (**f**). *Bars* **a**, **d** 40 μm; **b** 60 μm; **c**, **d**, **f** 20 μm

2008, L.S. Yadav; AMH 9279, in vitro culture no. NFCCI 1494.

Additional cultures examined: NFCCI 2199 isolated from partially degraded *T. chebula* fruits collected from Mahabaleshwar, Maharashtra, India, August 2009, L.S. Yadav; NFCCI 1496 (*G. simplex*), Mahabaleshwar, India, on decomposing fruits of *T. chebula*, Oct. 2008, L.S. Yadav.

Internal transcribed spacers (ITS) and 5.8S rRNA gene sequence similarity confirmed the identity of the isolates with >99% sequence homology with respective species. For comparison, rDNA sequences (ITS regions including 5.8S) of Indian isolates were aligned with authentic sequences available in the GenBank database including those from ex-type strains of all known *Gliocephalotrichum* species using CLUSTAL W. The neighbor-joining tree resulted in a monophyletic clade of *Gliocephalotrichum* with bootstrap value of 99% (Fig. 2). The *Gliocephalotrichum* clade formed three subclades: the first one consists of *G. bulbilium*, *G. cylindrosporum*, and *G. simplex*; the second subclade consists of *G. microchlamydosporum* and *G. ohiense*; and the third subclade consists of *G. bacillisporum* and *G. longibrachium*.

Strain NFCCI 1921 of *G. longibrachium* differed at four positions (2 deletions, 1 insertion, and 1 transversion) from the ex-type strain of *G. longibrachium* (MUCL 46693). The ex-type strain of *G. bulbilium* (MUCL 3185) differed from NFCCI 1494 in only one position in 478 nucleotides. Aligning sequences from Indian strains with those of authentic/ex-type strains, we recognized several species-specific variable nucleotide positions within the ITS1 and ITS2 region (TreeBase submission ID 12091).

The genus *Gliocephalotrichum*, established with the type species *G. bulbilium* J.J. Ellis & Hesselt., contains seven species described mostly from tropical or subtropical countries. The geographic distribution of this genus is Japan, Thailand, French Guiana, South Africa, Brazil, Malaysia, India, and the United States (Decock et al. 2006; Nishijima et al. 2002; Watanabe and Nakamura 2005; Haung and Schmitt 1973). The species concept of this genus is based on distinguishing characters, such as position of sterile arms on conidiogenous penicillus; sterile arms subtending to penicillus (*G. bulbilium*, *G. microchlamydosporum*, *G. ohiense*, *G. bacillisporum*, and *G. longibrachium*), and sterile arms arising at some distance below the penicillus (*G. simplex* and *G. cylindrosporum*). Nicot (1967)

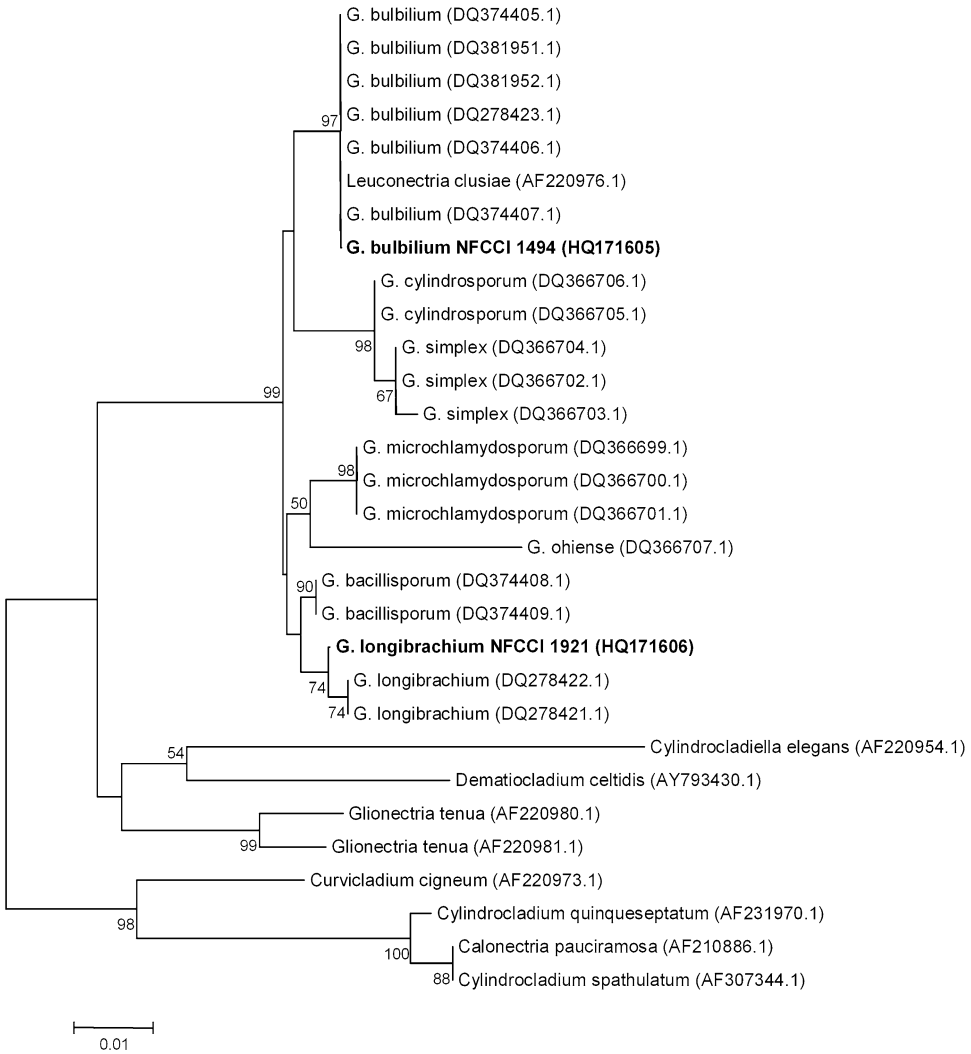

Fig. 2 Internal transcribed spacers (ITS) and 5.8S rRNA gene sequence-based phylogenetic relationships of *Gliocephalotrichum bulbilium* (NFCCI 1494) and *G. longibrachium* (NFCCI 1921) with other strains of *Gliocephalotrichum*. The phylogenetic tree was drawn using 610 nucleotides of ITS 1, 2, and 5.8S rRNA gene sequences using the neighbor-joining method in MEGA v.5 software. *Bar* represents the distances calculated in MEGA; *values at nodes* indicate the bootstrap percentages. Bootstrap values less than 50% are not shown

noticed some variability in location of the sterile arms, especially in *G. bulbilium.* The isolate of *G. bulbilium* obtained in the present study showed differences in the characters from the type description of the species. They differed in growth rate (80 mm in 3 days vs. 80 mm in 10 days), hyphal/sterile arms (numbers: 2–9 vs. 1–4 or more; size: up to 550 vs. 100–250 µm), conidiophores (185–575 vs. 160–340 µm), chlamydospores (35–112 vs. 50–90 µm), metulae, and conidia. Variation in the morphology of *G. longibrachium* was also recorded when compared with the original type description of *G. longibrachium* (Decock 2006). Conidiophores were longer (140–225 µm) in the Indian isolates and conidia smaller (5–8.5 µm) than in the French Guiana isolates (Decock et al. 2006). The aggregated chlamydospore cells form different shapes.

Soil and leaf litter (Wiley and Simmons 1971; Haung and Schmitt 1973; Decock et al. 2006; Jalmi et al. 2011) have been reported to be the habitats for species of *Gliocephalotrichum*. Arya (1994) reported *Gliocephalotrichum* on decayed fruits. In our studies we observed *Gliocephalotrichum* predominantly colonizing *T. chebula* fruits, suggesting the possibility of selective invasion and development on these fruits. Search for members of the genus on other fallen fruits collected in the same locality proved negative. It appears worthwhile to focus on mycobiota of decomposing fruits in contact with litter to identify novel and unique taxa. As such, our study on decomposing fruit in which we obtained three *Gliocephalotrichum* species on the same fruit indicates that fruit litter may harbor specialized fungal flora involved in decomposition.

Natural fungal resources are considered to contribute a great deal to biotechnological applications (Hoffmeister and Keller 2007). The biotechnology application potential of *Gliocephalotrichum* has not been explored, except in some reports indicating them as a source of melanin for use in plastic laminates for optical applications (Gallas and Eisner 2006) and in skin care formulations (Jalmi et al. 2011). It would be desirable to explore the biodiversity of the genus from diverse habitats and explore them for

valuable secondary metabolites and industrial enzymes with novel applications. Work on standardization of various fermentation parameters, such as pH, temperature, aeration, and media composition for the production of melanin by *Gliocephalotrichum* spp. described in this article, is in progress, and preliminary results obtained are promising.

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