Resolving the phylogenetic placement of *Gangliostilbe* in the family *Xenospadicoidaceae* (*Xenospadicoidales*)

Sneha Lad[1], Kunhiraman C. Rajeshkumar[1, *], Paraparath O. Sruthi[1], Nikhil Ashtekar[1], Abdulsalim P. Ansil[1], Rajnish K. Verma[2], Nalin N. Wijayawardene[3]

[1]National Fungal Culture Collection of India (NFCCI), Biodiversity and Palaeobiology (Fungi) Group, MACS Agharkar Research Institute, G. G. Agarkar Road, Pune, 411 004, Maharashtra, India

[2]Department of Plant Pathology, Punjab Agricultural University, Ludhiana, 141 004, Punjab, India

[3]Center for Yunnan Plateau Biological Resources Protection and Utilization, College of Biological Resource and Food Engineering, Qujing Normal University, Qujing, Yunnan, 655011, P. R. China

[*]Corresponding author, email: rajeshfungi@gmail.com

Abstract

This study resolves the phylogenetic position of an anamorphic synnematous genus, *Gangliostilbe*, (typified by *G. indica*) collected from the Northern Western Ghats of India. *Gangliostilbe* can be distinguished based on its erect synnemata, simple, dark-coloured, well-defined stalk, a subglobose to clavate head, and solitary, acrogenous, brown, phragmoconidia having three or more septa that secede through gangliar conidiogenesis. Phylogenetic analysis based on ITS and LSU sequence data supported the placement of *Gangliostilbe* in *Xenospadicoidaceae* (*Xenospadicoidales*). *Gangliostilbe* is the only genus in *Xenospadicoidaceae* having synnematous conidiomata and gangliar conidiogenesis.

Keywords: anamorphic ascomycota, phylogeny, synnemata, taxonomy, Western Ghats

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Introduction

Northern Western Ghats have a rich and diverse mycobiota, especially the asexual ascomycetes (Singh et al. 2010, Rajeshkumar and Singh 2012, Rajeshkumar and Sharma 2013, Rajeshkumar et al. 2018, 2019, 2021). During the surveys conducted to explore the microfungal diversity of Northern Western Ghats of Tamhini village and adjacent areas in the monsoon season of 2019, a synnematous fungus was collected. Extensive morphological studies confirmed that the new collection is *Gangliostilbe indica* Subramanian & Vittal, the type of *Gangliostilbe* Subramanian & Vittal (Vittal 1975). It has key distinguishing characters such as solitary, obovoid, ovoid or fusiform conidia borne on integrated, terminal, monoblastic conidiogenous cells on synnematous conidiophores. Later, three species were added to the genus, *viz.*, *G. costaricensis* Mercado et al., *G. malabarica* Subram. & Bhat, and *G. verrucosa* Bhat & B. Sutton (Bhat and Sutton 1985, Subramanian and Bhat 1987, Mercado Sierra et al. 1997). In addition, Ma et al. (2014) further introduced *G. yunnanensis*, characterized by synnematous conidiophores with monoblastic conidiogenous cells producing obovoid to ellipsoidal, 6- or 7- distoseptate conidia. Species of *Gangliostilbe* are mainly differentiated based on conidial characters such as size, shape, septation, ornamentation and pigmentation. In Index Fungorum (accessed on 12/12/2021), *Gangliostilbe* is currently classified under *incertae sedis*, *Pezizomycotina*, *Ascomycota*. Wijayawardene et al. (2017, 2020) placed the

genus under *Ascomycota* genera *incertae sedis*. This study aims to resolve the phylogenetic placement of this synnematous fungus in the fungal tree of life.

Materials and methods

Isolation

Conidia were isolated directly from a decaying litter and observed using a Nikon binocular stereomicroscope (Model SMZ-1500 with Digi-CAM, Japan). Single conidial cultures were established on 2 % malt extract agar (MEA) medium, following the method described in Rajeshkumar et al. (2021). Zeiss (AXIO Imager 2, Germany) and Olympus (Model CX-41, Japan) microscopes were used for morpho-taxonomic studies and photomicrographs. Conidia and conidiophores were mounted in lactic acid cotton blue and measured using an ocular micrometer (and confirmed with software available with the Zeiss microscope), with 30 observations per structure. Colony characteristics of the cultures were studied on MEA and potato dextrose agar (PDA) media. Colony colours and pigmentations were determined using Methuen's Handbook of Colour (Kornerup and Wanscher 1978). Herbarium specimens are maintained in RKC-LAB-B#36 collections and cultures were preserved at NFCCI; WDCM-932, Agharkar Research Institute, Pune, India.

Morphological study

Colony characters were recorded after 7 days of incubation on various media, including PDA, MEA (HiMedia, India). For inoculations, incubation conditions and microscopic slide preparations, the work of Senanayake et al. (2020) was followed. Colour codes and names used in descriptions refer to Kornerup and Wanscher (1978). Microscopic observations were noted with an Olympus (Model CX-41, Japan) dissecting microscope and Zeiss (AXIO Imager 2, Germany) compound microscope equipped with Nikon Digital sight DS-Fi1 and AxioCam MRc5 cameras driven by AxioVision Rel 4.8 software (AXIO Imager 2, Germany).

DNA extraction, amplification, and phylogenetic analyses

Colonies were grown on MEA medium, and genomic DNA extraction was done following the modified protocols of the rapid salt extraction method of Aljanabi and Martinez (1997). Internal Transcribed Spacer (ITS) and Large Subunit (LSU) 28S rRNA gene regions were amplified using the primer pairs ITS5 with ITS4 (White et al. 1990) and LROR with LR7 (Vilgalys and Hester 1990), respectively. The amplification was performed in a 25 µl reaction volume containing 9.5 µl ddH_2O, 12.5 µl 2× Taq PCR Master Mix with blue dye (Sangon Biotech, China), 1 µl of DNA template and 1 µl of each primer (10 µM). The amplification condition for ITS and LSU consisted of initial denaturation at 94 °C for 3 min; followed by 40 cycles of 45 s at 94 °C, 50 s at 56 °C and 1 min at 72 °C and a final extension period of 10 min at 72 °C. The PCR products were purified with StrataPrep PCR Purification Kit (Agilent Technologies, TX, USA), and sequenced using the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequencing reactions were run on ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA).

Sequence alignment and phylogenetic analysis

Sequences with high % identities were determined from a BLAST search to find the closest matches with allied taxa. Sequences generated from ITS and LSU regions were analysed together with reference sequences retrieved from GenBank following Luo et al. (2019). The multiple sequence datasets were aligned with MAFFT v.7 at the webserver (http://mafft.cbrc.jp/alignment/server; Katoh et al. 2017), and manually edited where necessary in BioEdit v.7.0.9.0 (Hall 1999). The phylogeny website tool, ALTER (Glez-Peña et al. 2010) was used to transfer the alignment file into PHYLIP format for RAxML analysis. Phylogenetic analyses of both individual and combined aligned data

were performed with the maximum likelihood (ML) method. Maximum likelihood analysis was performed via RAxML v.8 on the CIPRES web portal (Stamatakis 2006, 2014, Stamatakis et al. 2008) as part of the "RAxML-HPC2 on XSEDE" (http://www.phylo.org/portal2/; Miller et al. 2010). RAxML rapid bootstrapping and subsequent ML search used distinct model/data partitions with joint branch length optimization, executing 1000 rapid bootstrap inferences and a thorough ML search thereafter. All free model parameters were estimated by RAxML and ML estimate of 25 per site rate categories. Likelihood of final tree was evaluated and optimized under GAMMA+P-Invar Model parameters. GAMMA Model parameters were estimated to an accuracy of 0.1000000000 log-likelihood units. Every 100th tree was saved. The resulting trees were illustrated with TreeView 1.6.6 (Page 1996) and tree layout was created in Microsoft PowerPoint. DNA sequences newly generated in this study were deposited in GenBank.

Results

Phylogenetic analyses

Based on a Mega BLAST search of the new strain using the ITS sequence in NCBI's GenBank nucleotide database, the closest hits were *Neospadicoides lignicola* MFLU 18-1606 (GenBank NR_168815.; Identities = 449/487(92 %), 3 gaps (0 %)), *Neospadicoides aquatica* MFLU 18-1605

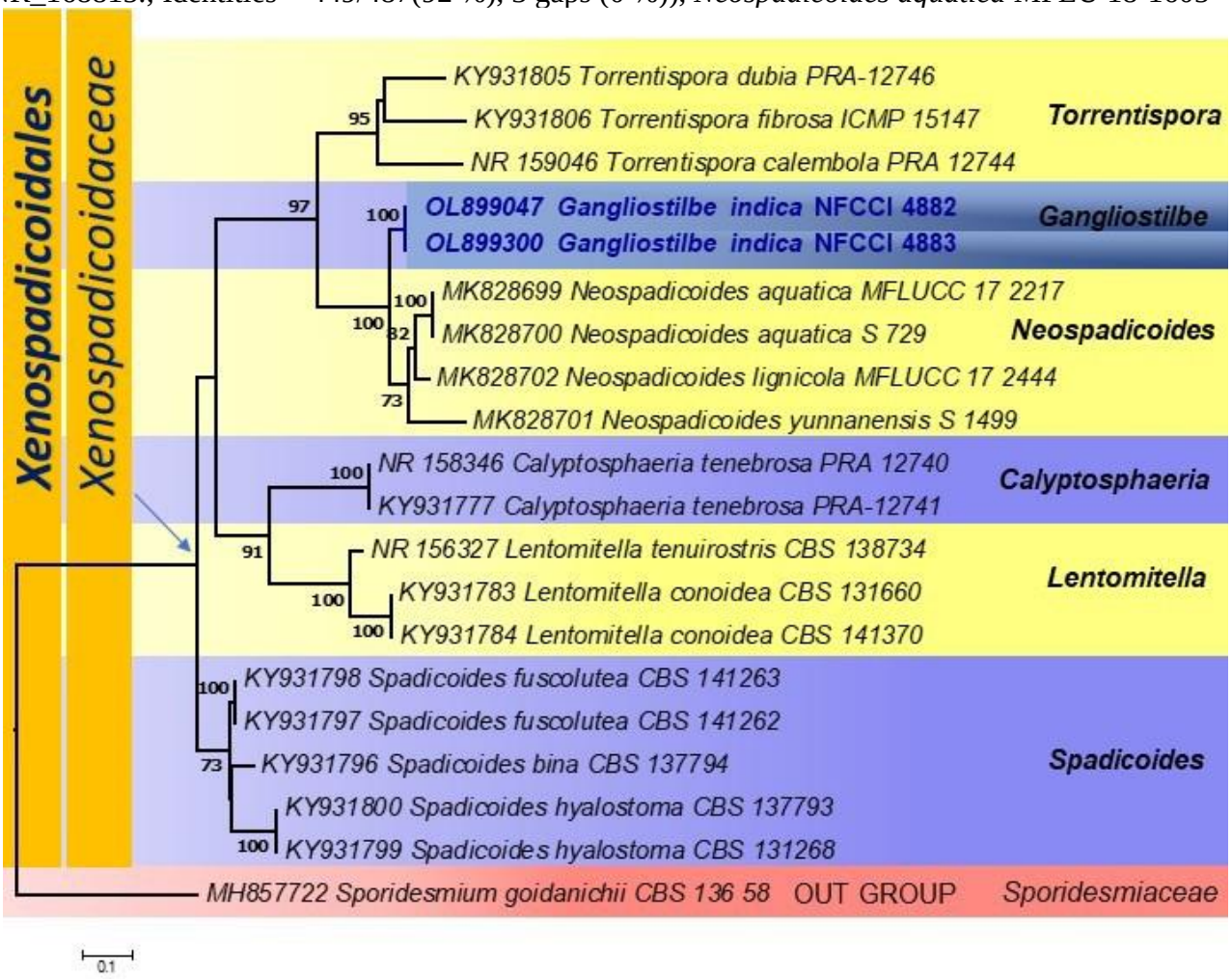

Fig. 1 The phylogram was generated from RAxML analysis based on ITS sequence data for the family *Xenospadicoidaceae* with a final likelihood value of -3989.865011. The matrix had 379 distinct alignment patterns. Estimated base frequencies were as follows: A = 0.233645, C = 0.281136, G = 0.259965, T = 0.225253; substitution rates: AC = 1.742771, AG = 4.179649, AT = 2.756792, CG = 1.228638, CT = 7.524084, GT = 1.000000; gamma distribution shape parameter α = 1.055122. Bootstrap support values for ML, ≥70% are given above the nodes. The tree is rooted to *Sporidesmium goidanichii* (CBS 136.58). Sequences generated in this study are shown in bold and blue.

(GenBank NR_168813; Identities = 446/483 (92 %), 3 gaps (0 %)), and *Neospadicoides aquatica* MFLUCC 17-2217 (GenBank MK828699; Identities = 446/483 (92 %), 3 gaps (0 %)). A similar search using the LSU sequences showed highest similarity to *Neospadicoides aquatica* MFLUCC 17-2217 (GenBank MK849851; Identities = 695/709 (98 %), 3 gaps (0 %)), *Neospadicoides lignicola* MFLUCC 17-2444 (GenBank MK849854; Identities = 674/685 (98 %), 0 gap (0 %)). The Mega BLAST and multigene phylogenetic analyses further supported the placement of the *Gangliostilbe* collected from the Northern Western Ghats of India within the family *Xenospadicoidaceae*. The phylogenetic relationships between the new strain and other accepted genera in *Xenospadicoidaceae* and their genetic congruence and phylogenetic consistency were analysed and interpreted using ITS and LSU sequence datasets (Figs. 1, 2). Both ITS and LSU datasets confirmed the placement of *Gangliostilbe* in *Xenospadicoidaceae*. *Gangliostilbe indica* formed a sister clade to *Neospadicoides* with strong support (100 % ML), based on ITS and LSU datasets. *Torrentispora* was also aligned as a sister clade with 97 % ML support in ITS phylogeny.

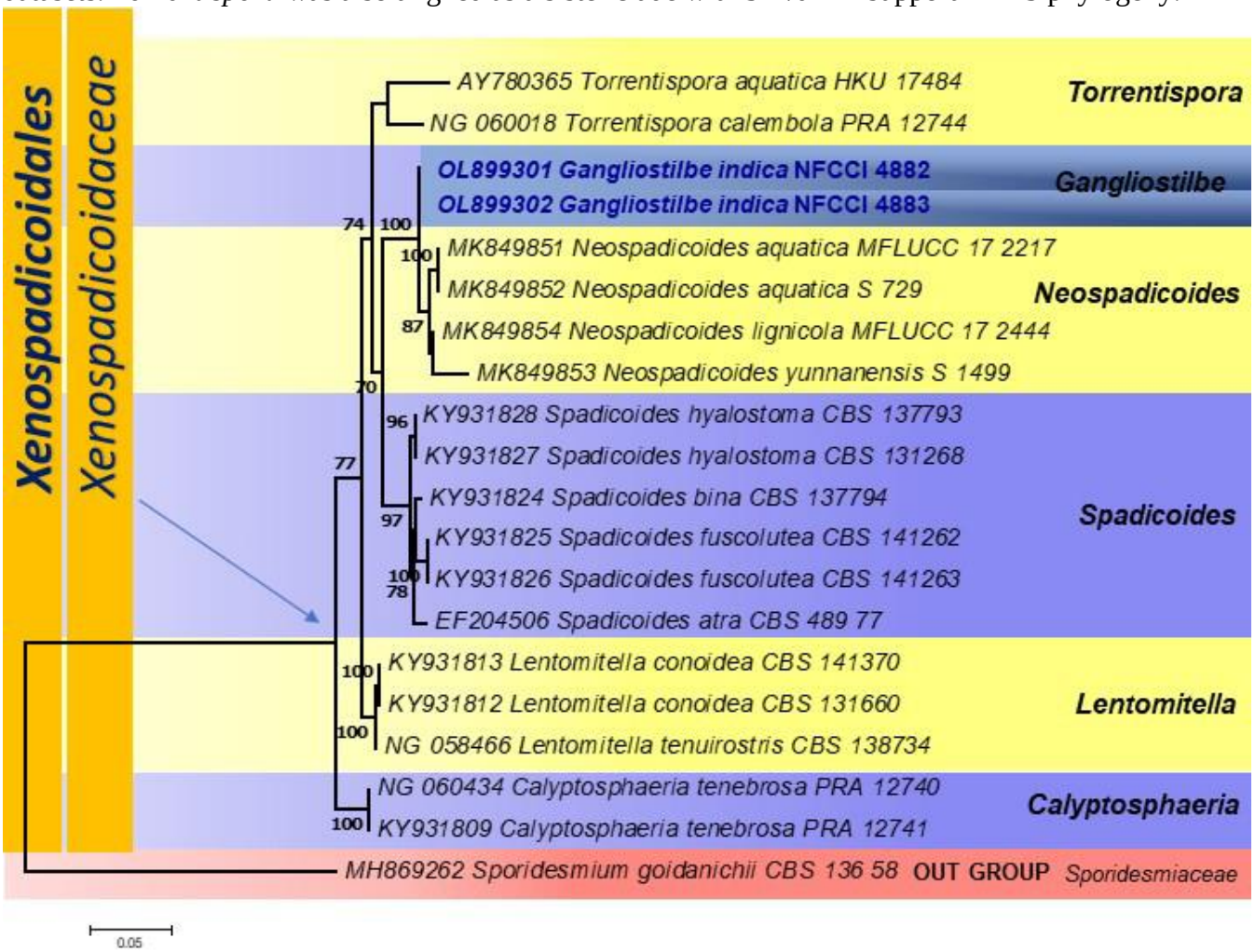

Fig. 2 The phylogram was generated from RAxML analysis based on LSU sequence data for the family *Xenospadicoidaceae* with a final likelihood value of -4326.521714. The matrix had 243 distinct alignment pattern. Estimated base frequencies were as follows: A = 0.259914, C = 0.224202, G = 0.294203, T = 0.221680; substitution rates: AC = 0.246057, AG = 1.322944, AT = 0.990044, CG = 0.167629, CT = 5.457432, GT = 1.000000; gamma distribution shape parameter α = 0.622610. Bootstrap support values for ML, ≥70% are given above the nodes. The tree is rooted to *Sporidesmium goidanichii* (CBS 136.58). Sequences generated in this study are shown in bold and blue.

Taxonomy
Gangliostilbe Subram. & Vittal, Kavaka 3: 70, 1976 (1975).

Index Fungorum Registration Identifier: IF 8309. Mycobank MB8309

Saprobic, *Conidiomata* synnemata, erect, simple, dark coloured, each with a well-defined stalk and a subglobose to clavate head. Individual hyphae of the synnema become free in the stalk's fertile part, diverging from the stalk to form conidiophores. *Conidiophores* simple, brown, septate, producing phragmoconidia. *Conidiogenous cells* integrated, terminal, monoblastic, cylindrical. *Conidia* solitary, gangliar, acrogenous, brown, 3 or more septate.

Type species: *Gangliostilbe indica* Subramanian & Vittal

Gangliostilbe indica Subram. & Vittal, Kavaka 3: 70 (1976) [1975]

Sexual morph: unknown. **Asexual morph**: Colonies on natural substrate form synnematous conidiomata. Mycelium superficial or immersed, composed of branched, septate, brown, smooth hyphae, 2–3 µm wide. *Conidiomata* synnemata, erect, unbranched, with dark brown stalks, consisting of compact aggregation of parallel conidiophores, terminating in brown fertile heads, up to 600 µm long, 25–40 µm wide, up to 60 µm wide at the base. *Conidiophores* unbranched, septate, cylindrical, smooth, brown, 5–7 µm wide, divergent towards the distal part of the synnema. *Conidiogenous cells* integrated, terminal, monoblastic, cylindrical, determinate, 9–15 × 4–6 µm. *Conidia* obovoid to ellipsoidal, truncate at the base, pale brown to brown, 3-septate, thin-walled, smooth, often guttulate in each cell, 24–40 × 10–15 µm.

Material examined: INDIA, Maharashtra State, Pune District, Tamhini village, on decaying wood, 9th July 2019, KC Rajeshkumar, Specimen No. NFCCI-RKC2019.01, Cultures NFCCI 4882, NFCCI 4883. GenBank accession numbers: NFCCI 4882-OL899047 (ITS), OL899301 (LSU); NFCCI 4883-OL899300 (ITS), OL899302 (LSU).

Discussion
Xenospadicoidales and *Xenospadicoidaceae* (Hern.-Restr. et al.) Reblova & A.N. Mill. were introduced by Hernández-Restrepo et al. (2017) and further emended by Reblova et al. (2018) to accommodate *Calyptosphaeria* Reblova & A.N. Mill, *Lentomitella* Höhn., *Spadicoides* S. Hughes (=*Xenospadicoides* Hern.-Restr. et al.; =*Pseudodiplococcium* Hern.-Restr. et al.) and *Torrentispora* K.D. Hyde et al. Reblova et al. (2018) determined the circumscription of the family *Xenospadicoidaceae* with the following key characteristics; Lignicolous, Ascomata perithecial, nonstromatic, with venter immersed, partially erumpent becoming superficial. Neck cylindrical or rostrate with or without sulcations. Ostiole periphysate. Hamathecium consists of septate, tapering paraphyses. Asci unitunicate, persistent, cylindrical or cylindrical-clavate, 8-spored, with a non-amyloid apical annulus. Ascospores hyaline or pale brown prior to discharge, aseptate or septate, variable in shape, smooth-walled or ornamented. Asexual morphs dematiaceous hyphomycetes producing effuse colonies. Conidiophores macronematous, mononematous, branched or unbranched. Conidiogenous cells tretic or holoblastic-denticulate, sympodially proliferating. Conidia hyaline or brown, aseptate or septate, variable in shape. In morphology, our new collection (NFCCI-RKC2019.01) was similar to the holotype of *Gangliostilbe indica* (Holotype MUBL 2345) (Vittal 1975). We, therefore, concluded that our collection is *G. indica.*

Recently, Luo et al. (2019) introduced a new genus *Neospadicoides,* representing an independent

lineage within *Xenospadicoidaceae*. In the present study, two isolates of *Gangliostilbe indica* (NFCCI 4882 and NFCCI 4883) accommodated in the same family. However, both strains form a sister clade to *Neospadicoides* spp. with high support in ITS and LSU molecular analysis (Figs. 1, 2). *Gangliostilbe* is also morphologically distinct from other genera in *Xenospadicoidaceae*, producing synnematous conidiomata and gangliar conidiogenesis, which are amended in the family circumscriptions by Reblova et al. (2018).

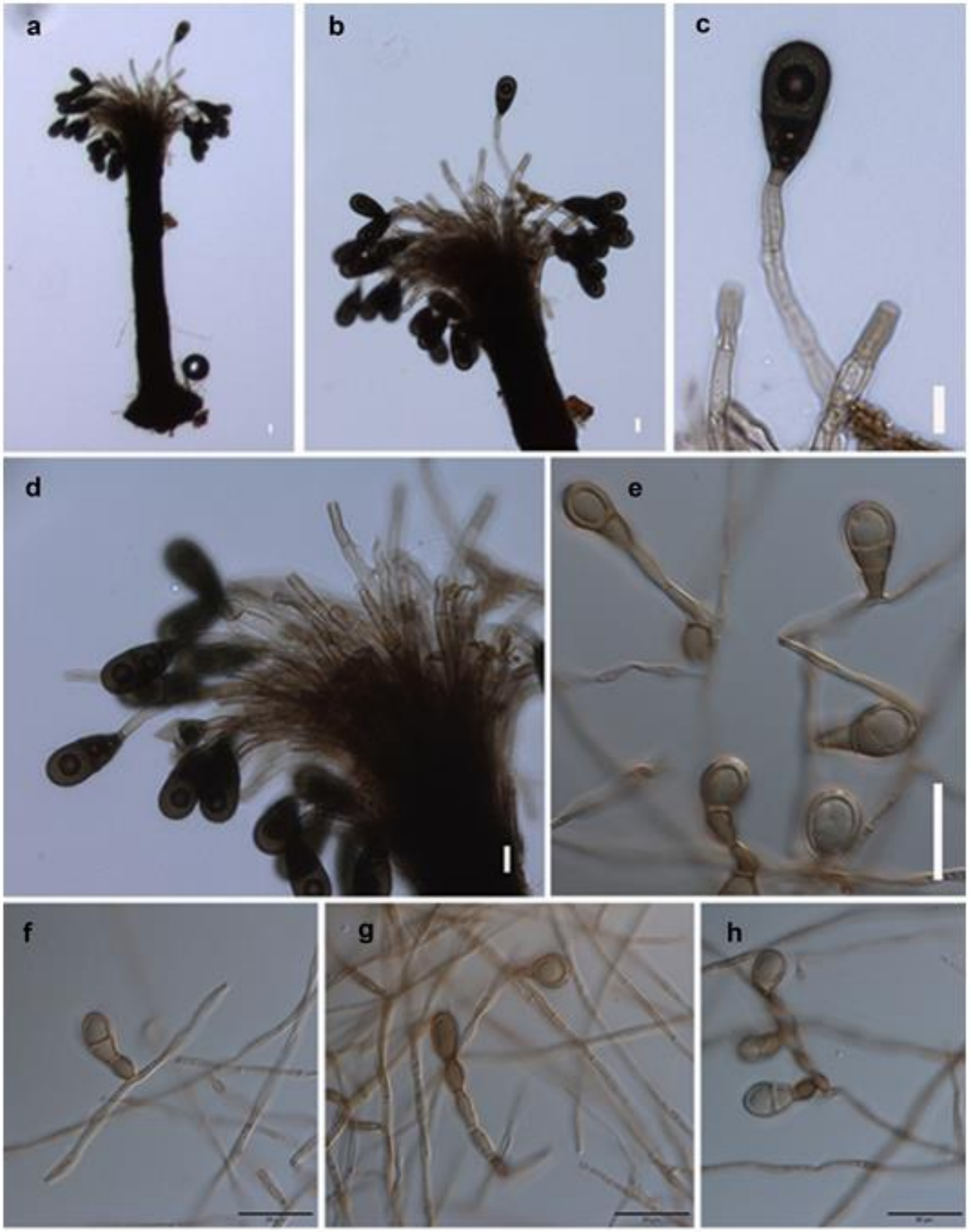

Fig. 3 ***Gangliostilbe indica*** **(NFCCI 4882). a** Synnematous conidiomata. **b** Fertile capitulum. **c** Conidiophore with mature conidia. **d** Divergent Conidiophores with developing and mature conidia. **e f, g, h** Conidial development in culture (NFCCI 4882). Bar is equal to 10 µm.

So far, five species of *Gangliostilbe* have been recorded as saprophytes from four different countries. The details of all the species records, host, holotype and countries are listed in Table 1. Two Indian species *G. malabarica and G. indica* were reported from the pristine habitats of the Southern Western Ghats, viz. Silent Valley, Kerala and Shimoga, Karnataka, respectively. Here, a key is provided (adapted from Ma et al. 2014) to distinguish *Gangliostilbe* species based on morphology. So far, only the type species has sequence data; thus, it is essential to recollect other species to determine the phylogenetic placements of the other species.

Table 1 Species records, host and country details of *Gangliostilbe*

***Gangliostilbe* species**	**Host /Type**	**Country**
G. costaricensis Mercado et al. (1997)	On fallen rotten wood, Holotype HAC (M) 9143	Costa Rica
G. indica Subram. & Vittal (1975)	On dead stems of *Bambusa*, Holotype MUBL 2345	Karnataka, India
G. malabarica Subram. & Bhat (1989)	On dead twigs Holotype FFSI No 4360	Kerala, India
G. verrucosa Bhat & B. Sutton (1985)	On dead twig, Holotype IMI 289552a	Kaffa, Ethiopia
G. yunnanensis L.G. Ma & X.G. Zhang (2014)	On dead branches of *Pistacia chinensis*, Holotype HSAUP H2031	Yunnan, China

Key to species of *Gangliostilbe* (reproduced from Ma et al. 2014)

1. Conidia obovoid to ellipsoidal 2
1. Conidia neither obovoid nor ellipsoidal 3
2. Conidia broadly obovoid, uniformly pigmented, 38–48×20–25 µm, 3-septate; conidiophores 5–7 µm wide .....*G. costaricensis*
2. Conidial cells unevenly pigmented; conidiophores less than 5 µm wide 4
3. Conidia ovoid to obclavate, 12.5–16 × 4.5–7.5 µm, 2–3(–4)-septate, dark brown, apical and subapical cells pale brown and verruculose; conidiophores unbranched .....*G. verrucosa*
3. Conidia fusiform, gangliar, elongated, up to 17-septate, light brown, smooth, 27–58 × 7–10 µm; conidiophores branched .....*G. malabarica*
4. Conidia 26–42 × 12–15 µm, 3-septate; conidiophores 3– 4.5 µm wide .....*G. indica*
4. Conidia 33–55 × 6.5–11 µm, 6- or 7-septate; conidiophores 2.5–4.2 µm wide ……..……...*G. yunnanensis*

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Statement on conflict of interest
The authors declare that there is no conflict of interest.

Author contribution

The work was conceived by the corresponding author and all the experimental work and fieldwork were executed by the first six authors. RKC and NA performed analyses. The manuscript was written and revised by RKC, NNW and RKV.

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