A new record of *Helicosporium* for India – *H. linderi* from Western Ghats

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Abstract: A new record of the helicosporous ascomycete *Helicosporium linderi,* collected on dead bark of *Eucalyptus*, from Western Ghats region, India, is described and illustrated based on morphology, sequence analysis and phylogeny of ITS and LSU regions of rDNA.

Zusammenfassung: Ein neuer Nachweis des helicosporen Schlauchpilzes *Helicosporium linderi*, der auf abgestorbener Rinde von *Eucalyptus* aus der Western Ghats Region in Indien gesammelt wurde, wird basierend auf Morphologie, Sequenzanalyse und Phylogenie der ITS und LSU Regionen der rDNA beschrieben und illustriert.

Helicomyces LINK, *Helicosporium* NEES and *Helicoma* CORDA are the earliest erected helicoid hyphomycete genera, which are well recognized and distinguished based on different parameters and characteristics, e.g.: (1) habitat features such as freshwater, marine, terrestrial, saprobic or parasitic; (2) conidium colour; (3) direction of coiling of the conidium, whether clockwise or counter clock-wise; (4) thickness of the conidial filament and septation; (5) looseness and tightness of coiling; (6) number of coils and size of the conidium; (7) cultural characteristics; (8) secondary characteristics such as synanamorph, secondary conidia, chlamydospore, appendages; (9) anamorph-teleomorph connection (GHAO & al. 2007). Most of the helicosporous hyphomycetes are saprobic; a few species of *Dichotomophthoropsis*, *Helicomina*, *Helicorhoidion* and *Trochophora* are known to be plant pathogens. Saprobic helicosporic fungal genera like *Helicosporium* and *Helicomyces* usually form an inconspicuous effuse, flaky, white or brown loose cottony layer on the substrate. Besides, overall taxonomy of helicosporous fungi relied mostly on morphological characters. DNA sequencing and phylogenetic analysis has been used only during the past two decades in order to resolve the taxonomic ambiguities.

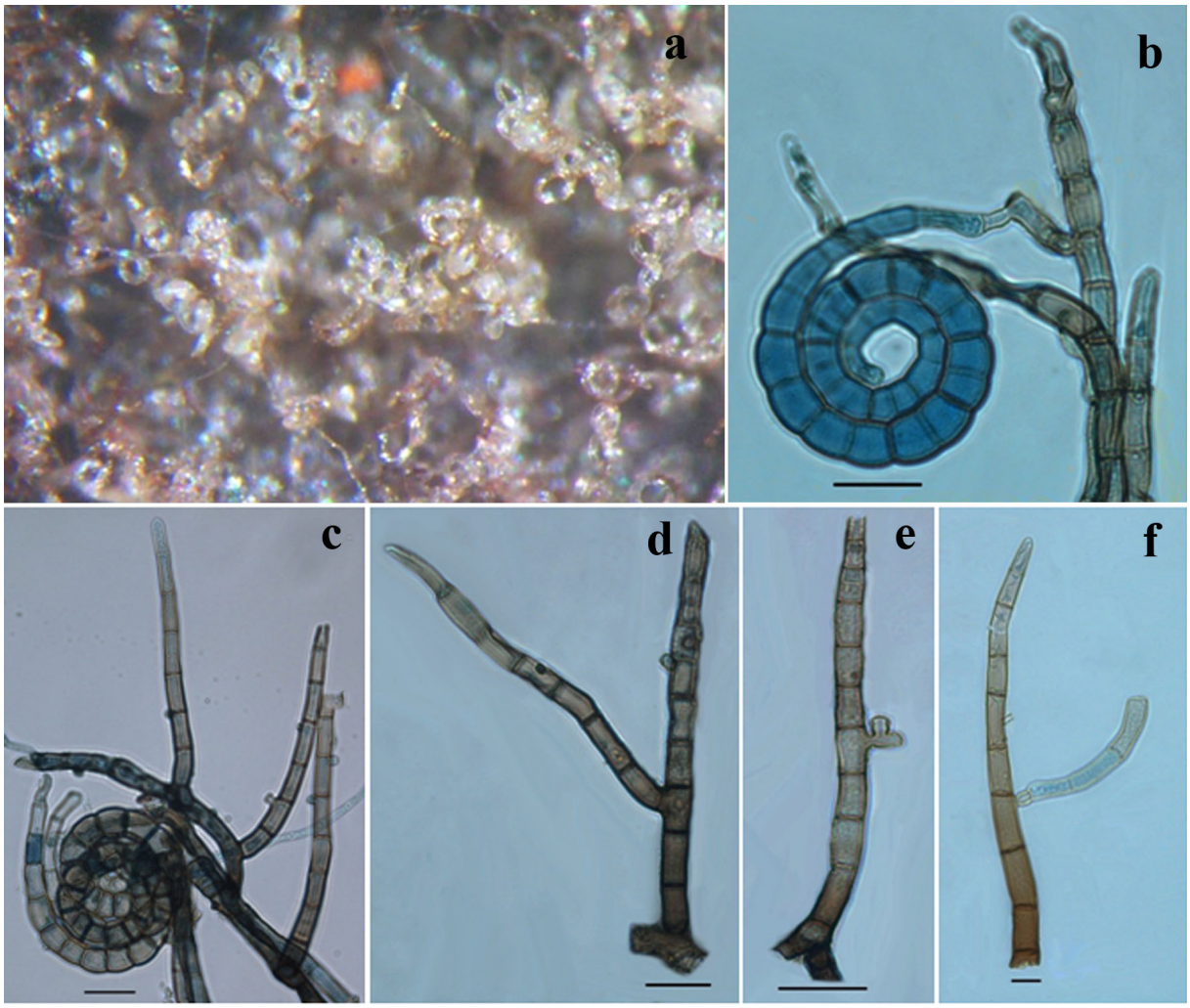

Fig. 1. *Helicosporium linderi* (AMH 9844). *a* Colonies on substrate. *b–c* Conidia and conidiophores. *d* Branched conidiophore with foot cell. *e* Unbranched conidiophore with denticulate to cylindrical conidiogenous cells. *f* Simple conidiophore attached with broken part of conidia. Bars: *a–e* 20 µm, *f* 10 µm.

In continuation of our fungal biodiversity exploration, documentation and conservation several microfungi have been collected, identified and recently reported from Western Ghats regions in India (SINGH & al. 2015, RAJESHKUMAR & al. 2016, SINGH & SINGH 2016). The fungal taxon in question was collected from the same geographic location. A detailed morphological and molecular study revealed it as *Helicosporium linderi* MOORE and as unknown from India (MANOHARACHARY & KUNWAR 2010).

Materials and methods

Isolates and morphology: The specimens were collected from Agharkar Research Institute premise, Western Ghats, India, and were brought to the laboratory. Presence of fungal patches on natural substrates were observed and located through a trinocular Nikon stereo microscope (Model SMZ-1500 aided with Digi-CAM) and photomicrographs were taken. Single spore isolation technique was applied and pure culture was successfully raised on potato dextrose agar (PDA). For colony colour identification, colour codes and terms are mostly taken from KORNERUP & WANSCHER (1978). Scrape mounts were also made in lactic acid and lacto-phenol cotton blue alone as well as in combination. The non-hygroscopic nature of conidia was confirmed using water. Microphotographs of various morphological structures were taken with a Zeiss AXIO-10 microscope. The specimen and culture

were deposited in the Ajrekar Mycological Herbarium (AMH) and in National Fungal Culture Collection (NFCCI).

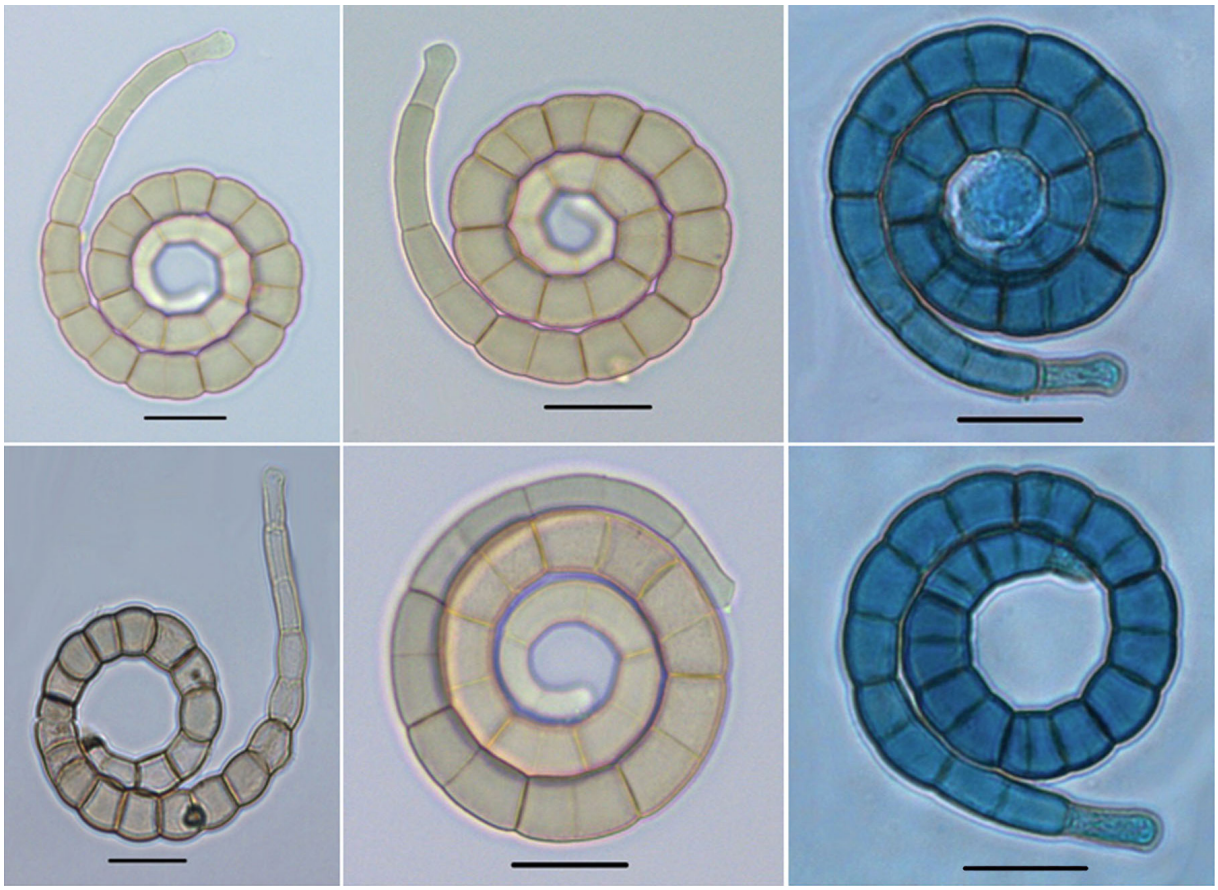

Fig. 2. *Helicosporium linderi*. Conidia mounted in lactophenol and cotton blue showing loose to compact, clockwise and anticlockwise coiling patterns. Bars 20 µm.

DNA extraction: DNA was extracted following AAMIR & al. (2015). Seven days old mycelia were placed in a 2 ml tube containing a ceramic pestle, 60–80 mg sterile glass beads (425–600 µM, Sigma) and lysis buffer (100 mM Tris HCl [pH8.0], 50mM EDTA, 3 % SDS). Homogenization was done twice in a FastPrep®-24 tissue homogenizer (MP Biomedicals, USA) at 6 M/S for 60 sec. The homogenate was centrifuged at 13000 rpm for 10 min and supernatant was transferred to a fresh microcentrifuge tube. To the supernatant, 2 µl of RNAse A (10 mg/ml) was added and incubated at 37 °C for 15 min. Then an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and mixed, followed by centrifugation at 13000 rpm for 10 min. The upper aqueous layer was taken in a fresh microcentrifuge tube and then the DNA was precipitated with 100 % ethanol. The DNA pellet was washed with 70 % ethanol and centrifuged at 12000 rpm for 5 min. The DNA pellets were air dried and dissolved in 1× TE buffer.

PCR and sequencing: The ITS and LSU region of rDNA were amplified by PCR using the primers ITS1 and ITS4 and LR0R and LR7, respectively (WHITE & al. 1990). The PCR products were purified with Axygen PCR cleanup kit (Axygen Scientific Inc., USA). Cycling of the PCR products was accomplished with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), using the PCR primers. The cycle sequencing products were run on an ABI Avant 3100 automated DNA sequencer (Applied Biosystems, USA). DNA sequences obtained were manually edited using ChromasLite v. 2.01 (http:/www.technelysium.com.au) and deposited in Gen-Bank (KY694684, KY694685).

Phylogenetic analysis: The ITS and LSU-rDNA sequences of the NFCCI isolate-4080 were subjected to BLASTn sequence homology searches. On the basis of the BLASTn search results, phylogenetically related species were chosen for the construction of the phylogenetic trees (separately for ITS and LSU-rDNA), which included different species of *Helicosporium* and *Tubeufia* (Tab. 2).

Cenococcum geophilum, Helicoma isiola and *Helicodendron conglomeratum* were chosen as outgroup. Multiple sequence alignment was performed with CLUSTAL W (http://www.ebi.ac.uk/clustalw/) and the phylogenetic analysis was performed by using the Maximum Likelihood method based on the Kimura 2-parameter model in MEGA 6.0 (KIMURA 1980, TAMURA & al. 2013). Optimal ML trees were found by a nearest neighbour interchanges (NNI) search, starting with a tree topology generated by the BIONJ method (GASCUEL 1997) using maximum composite likelihood (MCL) distances (TAMURA & al. 2004). One-thousand bootstrap replicates were analyzed to obtain nodal support values. The multiple sequence alignment used for construction of phylogenetic trees was submitted to TreeBASE (submission ID: 20752) with the Reviewer access URL: http://purl.org/phylo/treebase/phylows/study/TB2:S20752?x-access- code=692fea006c8b035c58d5b9e0a74b80be&format=html.

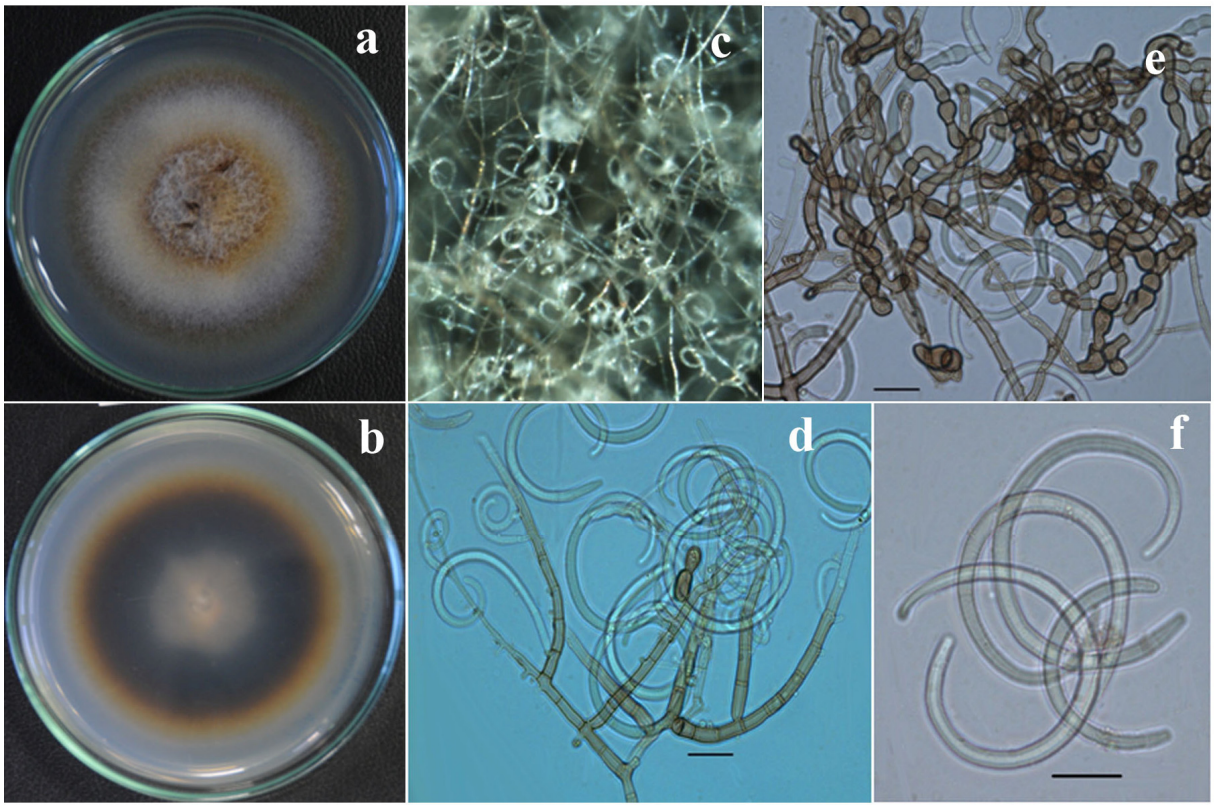

Fig. 3. *Helicosporium linderi* (NFCCI 4080). *a–b* Colony characteristics (front and reverse view on PDA. *c* Conidia and vegetative hyphae. *d* Conidiophores with arcuate to coiled conidia. *e* Vegetative hyphae, conidiophores and conidia. *f* Conidia. Bars 3–6 = 20 µm.

Description of the Indian specimen of *Helicosporium linderi.* – Figs. 1–5.

Characters:

Habit: Asexual colonies on inner bark surfaces, effuse, tan brown, velvety.

Conidiophores: macronematous, mononematous, erect, straight to slightly flexuous, septate, dark brown (6F8), smooth-walled, unbranched to branched, stiff, 70–303 × 8–9 µm, 4–22 septate.

Conidiogenous cells: integrated, terminal to intercalary, mono- to polyblastic, simple to branched, cylindrical to denticulate, 0–1 septate, olive brown (4E5), 5–16 × 3.5–6.5 µm.

Conidia: solitary, dry, smooth-walled, non-hygroscopic, acropleurogenous, simple, helicoid, olive brown (4E5), 23–30 septate, 43–69 × 40–62 µm diam. Conidial

filament 7.5–13.5 µm thick, base truncate sometimes obliquely truncate, constricted near septa, loosely as well as tightly coiled 1½–3½ times.

C o l o n i e s : on malt extract agar (MEA) growing slowly at 25 °C, reaching 29 × 29 mm diam. after 16 days, velvety to floccose, greyish brown (6E3), reverse greyish brown (6E3), margin irregular, abundantly sporulating with prolonged incubation duration. Hyphae septate, wall thickened and darkened, smooth-walled, constricted near septa, dark brown (6F8), 3.5–12 µm wide. Conidiophores arising from superficial hyphae, branched to unbranched, macronematous, multiseptate, olive brown (4E5), smooth walled, up to 240 × 7.15 µm. Conidiogenous cells acropleurogenous, denticulate to cylindrical, 4.5–5 × 2.5–3 µm. Conidia solitary, dry, smooth-walled, variable in shape and size, horse-shoe shaped, arcuate, vermiform and coiled 1–1½ times, septa not prominent, olive (2F6), 52–158.5 × 4–6.25 µm.

Tab. 1. Comparison of diagnostic characteristics of *Helicosporium linderi* NFCCI 4080 recorded from India with allied *Helicosporium* species.

***Helicosporium* species**	**Conidiophore size** (µm)	**Conidial diam.** (µm)	**Conidial coils**	**Conidial septation**	**Conidial filament width** (µm)
H. abuense	95–100 × 5.5	12–24	2–3½	6–18	2–2.75
H. decumbense	75–200 × 4–5	6–9	1–2	-	0.75–1.5
H. gracile	150 × 2.5–5	10–15	3–3¼	-	1–1.5
H. griseum	- × 3.5–5	18–25	2–4	10–14	1–2.5
H. guianense	480 × 2.5–4.5	20–22	3–3½	-	1–5
H. hiospiroides	150–250 × 5.5–8.5	21–35	1–4	1–4	1.5–3
H. indicum	19–43 × 3–5	25.2–36	1½–3½	5–12	1.4–2.5
H. lumbricopsis	-	20–28	3–4	18–25	1.5–2.5
H. nizamabadens	57–198 × 3–5	18.2–28	2–3½	15	1.4–2.2
H. panacheum	40–70 × 4.5–6	(15)20–30	2–4	Multiseptate	2.5–4.5
H. raghuveeri	80–170 × 6–8.5	60–85	1½–2½	20	3.5–7
H. vegetatum	600 × 3–6	10–20	2–4	14-19	0.8–1.3
H. myrtacearum	80-175 × 3.75–8	13–18.5	2–2½	8–12	1.75–2
H. xylophilous	16–65 × 4–6.25	13.75–40	1½–2½	17–24	3.5–4.25
H. linderi	70–303 × 8–9	43–69	1½–3½	23–30	7.5–13.5

Habitat, host plants and distribution: Dead bark of *Eucalyptus* (India) and on decaying leaves of *Cocos nucifera* (Panama, MOORE 1954, TSUI & al. 2006a).

Material examined: India, Maharashtra, Pune, Agharkar Research Institute campus (18° 31' N, 73° 55' E), on dead bark of *Eucalyptus* (*Myrtaceae*), 12. September 2016, P. N. SINGH, AMH 9844, PNS-ARI-19, culture deposited in National Fungal culture Collection of India (WDCM 932, NFCCI 4080).

Discussion

The overall comparison of the set of morphological characters (Tab. 1) as well as the ITS and LSU rDNA based phylogenetic analysis (Tab. 2, Figs. 4, 5) of our specimen are in accordance with *Helicosporium linderi*. In *H. linderi* the conidiophores are erect

Tab. 2. Sequences used for phylogenetic analysis.

Taxon	GenBank Accession No.	
	ITS	LSU
Cenococcum geophilum	KC967408	-
Helicodendron conglomeratum	-	AY856900
Helicosporium abuense	AY916470	AY916085
H. aureum	AY916478	AY856894
H. gracile	AY916485	AY916086
H. griseum	AY916474	AY856902
H. griseum	-	AY856884
H. guianense	AY916487	AY856893
H. indicum	AY916477	AY856885
H. linderi	AY916454	AY856895
H. linderi NFCCI 4080	KY694685	KY694684
H. lumbricoides	AY916476	AY856889
H. pallidum	AY916462	AY856913
H. pallidum	FN394720	-
H. pallidum	AY916460	AY856886
H. panachaeum	AY916471	AY916087
H. talbotii	AY916465	AY856874
Tubeufia cerea	AY916488	AY856883
T. helicomyces	AY916461	AY856887

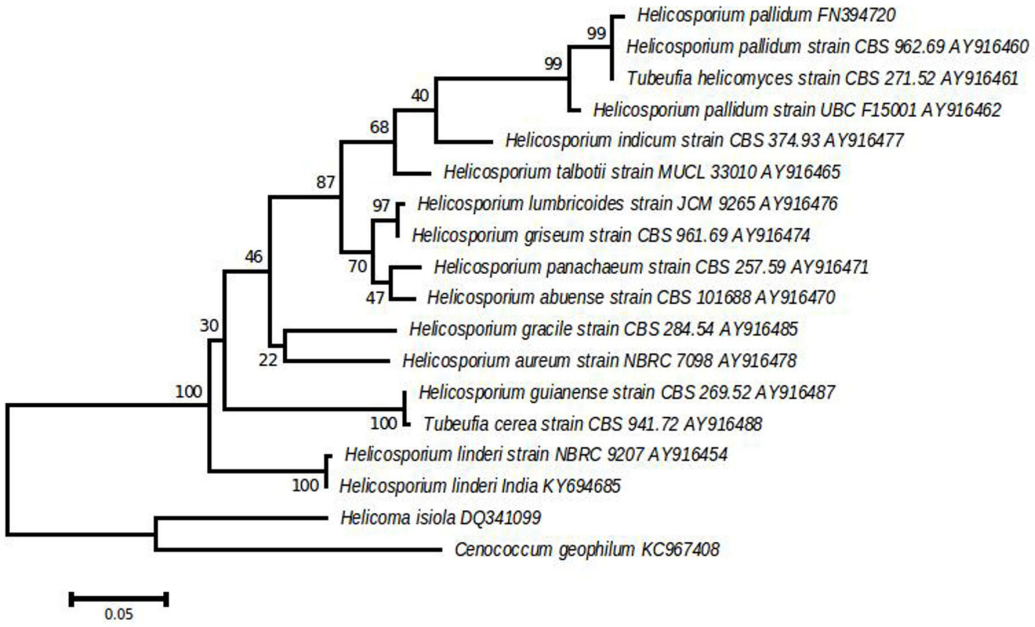

Fig. 4. Phylogram generated from ITS-rDNA sequences of *Helicosporium linderi* and allies. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [1]. The tree with the highest log likelihood (-2102.5647) is shown. *Cenococcum geophilum* and *Helicoma isiola* were considered as outgroup. Evolutionary analysis was conducted in MEGA6 (TAMURA & al. 2013).

and separate and the conidia pale brown. Though the conidia are reported to be hygroscopic in *H. linderi*, in our specimen they were non-hygroscopic. To our knowledge our Indian specimen is the second one known worldwide.

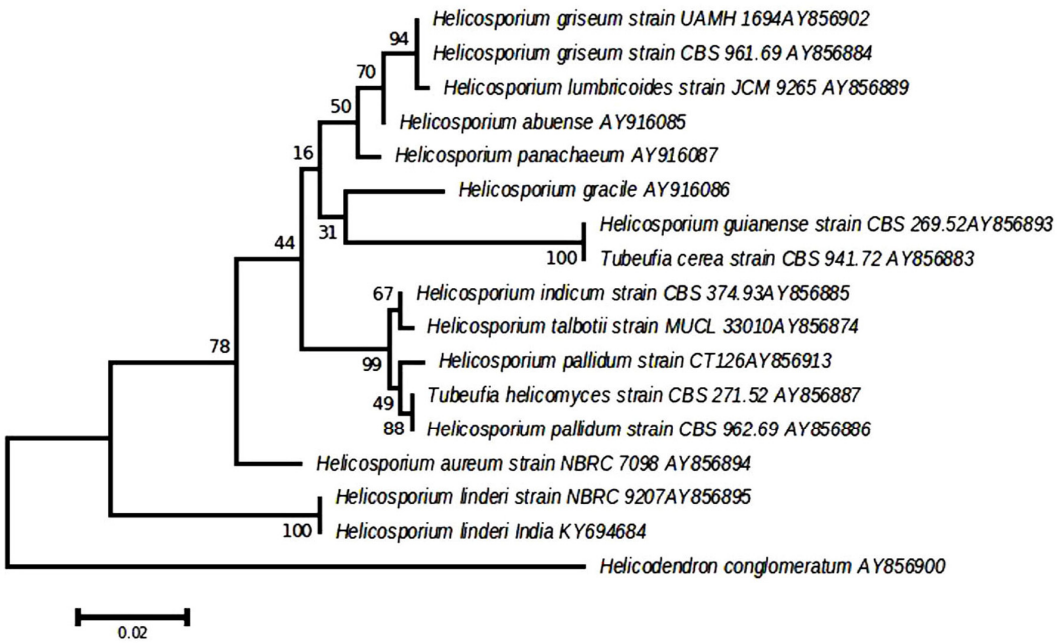

Fig. 5. Phylogram generated from partial LSU-rDNA sequences of *Helicosporium linderi* and allies. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [1]. The tree with the highest log likelihood (-1464.8822) is shown. *Helicodendron conglomeratum* was considered as outgroup. Evolutionary analysis was conducted in MEGA6 (TAMURA & al. 2013)

Phylogenetic relationships in these anamorphic helicosporous ascomycetes were inferred from ribosomal sequences from ITS and partial LSU regions and revealed a placement in the *Tubeufiaceae* (TSUI & al. 2006a, b).

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