**Tamhinispora** a new genus belongs to family *Tubeufiaceae* from the Western Ghats, India based on morphology and phylogenetic analysis

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A new genus and species, *Tamhinispora indica*, was collected on decaying *Bambusa bambos* culms from Tamhini Ghats, northern Western Ghats of Maharashtra. Morphologically, this new genus can be easily differentiated from similar genera like *Ernakulamia*, *Pseudoacrodictys*, *Petrakia*, *Biconiosporium*, *Pseudopetrakia* and *Manoharachariella* by having dark blackish brown, mostly ovoid or irregular, dictyoseptate conidia with apical appendages diverging or radiating from the conidial tip and intercalary, almost sessile conidiogenous cells in hyphae. Phylogenetic analysis using ITS and LSU sequences establish the placement of *Tamhinispora* in the family *Tubeufiaceae*; allied to dictyochlamydospore-forming or dark brown conidia-forming genera like *Chlamydotubeufia* and *Helicoon*.

**Key words** – anamorphic fungi – *Ascomycota* – dematiaceous – dictyoseptate – stauroconidium

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**Introduction**

The Western Ghats, a mega diversity hot spot situated in the southern west coast of the Indian Peninsula has a rich and diverse flora and fauna. The pristine natural forests, microhabitats, and tropical warm humid climate that prevail in the Western Ghats support many rare and new forms of fungi (Bhat & Kendrick 1993, Rajeshkumar 2007). During 2010–2012, surveys were conducted to explore the microfungal diversity in natural forests of southern and northern Western Ghats (Rajeshkumar et al. 2011a, b, Rajeshkumar & Singh 2012, Rajeshkumar et al. 2012). One of the survey in the bamboo dominating evergreen patch in the valley of the Tamhini Ghats resulted in the collection of a rare dematiaceous hyphomycete subsequently determined to be a new genus. The present study describes and illustrates this unusual dematiaceous, dictyoseptate, stauroconidium-forming hyphomycete collected from the Tamhini Ghats.
Methods

Isolates and morphology

Conidia were isolated directly from the surface of a dead twig and observed under a Nikon binocular stereomicroscope (Model SMZ-1500 with Digi-CAM, Japan). Single conidial cultures were established on 2% potato dextrose agar plates (PDA; Crous et al. 2009). For morphotaxonomic studies and photomicrographs, Zeiss (AXIO Imager 2, Germany) and Olympus (Model CX-41, Japan) microscopes were used. 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. Culture colony characteristics were studied on two different media: 2% malt extract agar (MEA) and PDA (Crous et al. 2009). Colony colours were determined using Methuen Handbook of Colour (Kornerup & Wanscher 1981). A herbarium specimen was deposited in the Ajrekar Mycological Herbarium (AMH), and the culture was accessioned and preserved at NFCCI; WDCM-932, Agharkar Research Institute, Pune, India.

DNA extraction, Polymerase chain reaction (PCR) and Sequencing – Total DNA was extracted from cultures grown on PDA plates for 2 weeks at 25°C, using the method of Aljanabi & Martinez (1997). Fragments containing the region encoding the 28S nrDNA (LSU) and ITS 1-5.8S nrDNA-ITS 2 (ITS) were amplified using primer pairs 5.8SR, LROR (Rehner & Samuels 1994) and LR7 (Vilgalys & Hester 1990) for LSU; ITS4 and ITS5 (White et al. 1990) for ITS. DNA amplification was performed in a 25 μl reaction using 2 μl of template DNA (25 ng), 1 U of Taq DNA polymerase (Genei, Bangalore, India), 2.5 μl of 10× Taq DNA polymerase buffer, 1 μl of 200 μM of each dNTPs (Genei, Bangalore, India), 1 μl of 10 pmol primer, H2O (Sterile Ultra Pure Water, Sigma) to make up 25 μl. Amplification in an Eppendorf Mastercycler Gradient 5331 AG used the following parameters: 5 min at 95°C; 30 cycles of 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C for the ITS region amplification; and final 7 min extension step at 72°C. DNA amplification of LSU followed the ITS conditions except for a 52°C annealing temperature. The PCR products were purified with an Axygen PCR cleanup kit (Axygen Scientific, CA, USA) and sequenced with the same primers using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequencing reactions were run on an ABI 3100 automated DNA sequence (Applied Biosystems, USA).

Sequence alignment and phylogenetic analysis – LSU and ITS sequences from Tamhinispora indica NFCCI 2924 were manually edited using Chromas Lite software (www.technelium.com.au) and deposited in the NCBI Genbank DNA sequence database (ITS: KC469282, LSU: KC469283). They were also subjected to a BLAST search of the NCBI nucleotide database. For phylogenetic analysis, the sequences were aligned using Clustal W together with the homologous regions of ITS and LSU of closely related genera and species. For construction of phylogenetic tree, the matrix was analyzed using Neighbor-Joining method of Molecular Evolutionary Genetics Analysis (MEGA) software v5.0. (Tamura et al. 2011). The sequence alignment and phylogenetic tree are deposited in TreeBASE (http://purl.org/phylo/treebase/Phylowsstudy/TB2:S13794). Spencermartinsia viticola (A.J.L. Phillips & J. Luque) A.J.L. Phillips, A. Alves & Crous (Phillips et al. 2008) belonging to family Botryosphaeriaceae was selected as out group.

Results

Tamhinispora Rajeshkumar & Rahul Sharma gen. nov.

MycoBank MB803105

Differs from the allied genera in having dark blackish brown ovoid to irregular conidia with radiating or diverging apical appendages from conidial tip and almost sessile conidiogenous cells.

Colonies effuse, blackish brown to black, Mycelium mostly semi-immersed or immersed, single or interwoven, Stroma none, Setae and hypopodia absent, Conidiophores absent, Conidiogenous cells, semi-macronematous, unbranched, intercalary in hyphae, almost sessile.
Conidia solitary, dry, simple, mostly ovoid or irregular, dictyoseptate, smooth, young conidia pale to dark brown with or without appendages, mature conidia dark brown to blackish with apical appendages. Apical appendages rudimentary or well developed, arising from tip of conidia, diverging or radiating, pale to dark brown, septe, tip rounded.

Type species

_Tamhinispora indica_ Rajeshkumar & Rahul Sharma, _sp. nov._ Figs 1–19 MycoBank 803106

Etymology – Genus named after the place of collection Tamhini Gaths and species named after the country where this fungus is native.

Colonies effuse, dark brown (6F4), blackish brown (6G8) to black. Conidia solitary, dry, simple, mostly ovoid or irregular, dark brown to blackish, dictyoseptate, paler towards tip when young, 54.5–108 × 34.5–50 µm, smooth, Apical appendages 0–9, rudimentary or well developed, arising from tip of conidia, radiating, pale to dark brown, septe, 0–7 septe, 13.5–95 × 3.8–6.3 µm.

Teleomorph – Unknown/Not observed.

Known distribution – found in the natural forests of northern Western Ghats.

Material examined – India, Maharashtra, Mulshi, Tamhini Gaths, on _Bambusa bambos_, 15 July 2012, Rajeshkumar KC, _holotype_, AMH 9555 – _ex-type_ culture in NFCCI 2924

Notes – The culture on MEA and PDA after 10 days at 25±2°C, brownish grey (6F2) to greyish brown (6E3) or blackish, slow growing, velutinous, 2–3 mm diam. after 60 days, centre becomes raised and dull reddish brown (8E8) after long incubation, reverse blackish, Sporulation not found in culture.

**Phylogenetic analyses**

Sequencing of the partial LSU gene of NFCCI 2924 resulted in 700 bp long sequence (KC469283) which in BLAST search showed maximum identity of 97% with _Helicomyces macrofilamentosus_ (HKUCC10235; AY849942.1), 96% with _H. roseus_ (BCC3381; AY78732.1 and AFTOL-ID 1613; DQ678083.1), _Chlamydotubeufia khunkornensis_ (MFLUCC10-0118; JN865190.1), _Tubeufia amazonensis_ (ATCC42524; AY87938.1), _Chlamydotubeufia huaiyangplaens_ (MFLUCC10-0926; JN865198.1), _T. cerea_ (IFO9014; AY849964.1), _T. paludosa_ CBS 120503; GU301877.1), 95% with _T. helicosporium_ (AFTOL-ID 1580; DQ767654.1), _T. cylindrotheca_ (BCC3559; AY849965.1), _Acanthostigma chiangmaiensis_ (MFLUCC10-0125; JN865197.1), _Thaxteriellopsis lignicola_ (AFTOL-ID 1580; DQ767654.1), _Helicosporium griseum_ (CBS 222.58; AY916469.1), _Helicomyces morganii_ (CBS 281.54; AY916468.1), _Helicosporium panachaeum_ (CBS257.59; AY916471.1), 89% with _Helicosporium griseum_ CBS 961.69; AY916474.1), _Helicomyces bellus_ (CBS 113542; AY916475.1), _Helicosporium lumbricoides_ (JCM 9265; AY916476.1) and 88% with several species belonging to _Helicosporium, Helicomyces_ and _Acanthostigma_. For phylogenetic analysis additional 25 sequences (21 species) belonging to family _Tubeufiaceae_ were retrieved from Genbank and aligned with the sequence of _Tamhinispora indica_. A Neighbor-joining tree was constructed from this analysis (Fig. 21) in which _Tubeufiaceae_ members formed four major clades. _Tamhinispora indica_ was placed in the second clade along with _Helicosporium panachaeum, Tubeufia aurantiella, Helicosporium griseum, Helicomyces violaceum_ and _H. morganii_, which are morphologically quite dissimilar from the new fungus.
Figs 1–9 – *Tamhinispora indica* (holotype). 1 Habitat Tamhini Ghats. 2, 3 Habit on *Bambusa bambos* decaying twigs. 4–7 Mature conidia in nature with rudimentary or well developed apical appendages. 8, 9 Single conidial cultures on PDA after 60 days.
**Figs 10–19** – *Tamhinispora indica* (holotype). **10** Conidial development. **11** Mature conidia with apical appendages. **12–19** Variation in conidial size, shape and apical appendages. – Bars = 20 µm.
Discussion

Considering the conidial morphology Tamhinispora can be classified under stauroconidium, body dictyoseptate with 3–5 radiating arms group (Seifert et al. 2011), which includes Ernakulamia Subram., Pseudoacrodictys W.A. Baker & Morgan-Jones and Petrakia Syd. & P. Syd. Another similar group with dictyoseptate conidia, dark, paler horns or lobes includes Biconiosporium Bat. & J.L. Bezzera, Pseudopetrakia M.B. Ellis and Manoharachariella Bagyan. N.K. Rao & Kunwar (Seifert et al. 2011). The conidia in Tamhinispora are mostly ovoid and dark brownish black or black in nature with radiating apical appendages, which are septate, pale brown and arising from the conidial tip.

Tamhinispora is unique and distinct from the morpho-taxonomically similar genera like Ernakulamia, Pseudoacrodictys, Petrakia, Biconiosporium, Pseudopetrakia and Manoharachariella. Morphologically Ernakulamia is most allied to Tamhinispora; both genera have reduced intercalary conidiogenous cells, diverging or radiating appendages and dictyoseptate conidia. However, conidial shape and arrangement of apical septate appendages are different. Conidia in Tamhinispora are mostly ovoid and apical appendages arise from tip of conidia, whereas in Ernakulamia conidia are irregular in shape and apical appendages arise from different conidial cells (various loci) of upper part in conidium. Furthermore, conidiogenesis is monoblastic and conidial secession is rhexolytic in Tamhinispora, in contrast to monotretic and schizolytic in Ernakulamia. In Pseudoacrodictys, conidiophores are well developed, unbranched, brown with percurrent proliferations; similarly, the genus Petrakia has sporodochia/stromata and conidiophores that evidently differentiate both these genera from Tamhinispora. Biconiosporium also resembles Tamhinispora with both genera possessing intercalary, monoblastic conidiogenous cells in hyphae. In contrast, Biconiosporium is different by short, non-septate apical arms. However, apical appendages in Tamhinispora are well developed, septate and diverging or radiating from conidial tip. Pseudopetrakia has reduced branched or unbranched conidiophores which are absent in Tamhinispora; in addition 2–4 black, sharp apical spines are unique in Pseudopetrakia. In Manoharachariella, conidia are apiculate and tiered and never have apical appendages; similarly conidiophores are well developed and branched which is reduced to intercalary, monoblastic conidiogenous cells in Tamhinispora.

Phylogenetic analyses of isolate NFCCI 2924 established the placement of Tamhinispora in family Tubeufiaceae. The Tubeufiaceae sensu Barr (1979) includes the type genus Tubeufia and was placed under Pleosporales. Most taxa of Tubeufiaceae are saprobic on dead plant material, especially wood, fungi or scale insects (Barr 1980, Rossman 1987, Kodsub et al. 2006, Promputtha & Miller 2010, Sánchez & Bianchini 2010). The Tubeufiaceae have been thoroughly studied and revised by several authors (Barr 1980, Rossman 1987, Kirk et al. 2001, Lumbsch & Huhndorf 2010) and there was a recent morphological re-examination and phylogenetic placement of major taxa from Thailand (Boonmee et al. 2011). Boonmee et al. (2011) made a definite progress towards establishing the generic concepts in the Tubeufiaceae and defined five distinct genera based on morphology and molecular data and accepted a further 14 genera based on morphology. The anamorphs of Tubeufiaceae have been well studied and include, helicosporous, staurosporous or dictyosporous forms belonging to Annellospermoporella, Aquaphila, Araneomyces, Guelichia, Helicoma, Helicoon, Helicomycyes, Helicosporium, Monodictys-like, Pendulispora, Peziotrichum, Tetraecrum, Titaea and Xenosporium (Ellis 1971, 1976, Kirk et al. 2008, Hyde et al. 2011). Phylogenetic analysis of larger ribosomal subunit of Tamhinispora indica shows Chlamydotubeufia Boonmee & K.D. Hyde (Boonmee et al. 2011) and Helicoon Morgan are close to Tamhinispora. Anamorphic states of both these genera form dark brown pigmented helicoids (in Helicoon) and dictyochlamydospores that somewhat resemble the conidia of the new genus. On the contrary, dark blackish brown, ovoid or irregular, dictyoseptate conidia with apical appendages radiating from the conidial tip is unique in Tamhinispora. Xenosporium Penz. & Sacc. is another dictyosporous anamorph included in the
Fig 20 – Phylogenetic tree derived from aligned LSU sequences of *Tamhinispora indica* inferred using the Neighbor-Joining method based on the recent generic concepts of the *Tubeufiaceae* established by Boonmee et al. (2011). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 448 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

*Tubeufiaceae* (Kirk et al. 2008, Hyde et al. 2011). *Thaxteriellopsis* is the teleomorph for *Xenosporium* species (Index Fungorum). Boonmee et al. (2011) epitypified the type species of *Thaxteriellopsis*, *T. lignicola* Sivan., Panwar & S.J. Kaur which forms a distinct clade in the phylogenetic tree and is justified as a genus distinct from *Thaxteriella*. Earlier studies by Subramanian & Sekar (1982) stated that *Thaxteriellopsis lignicola* was associated with *Moorella* like anamorphs. However, the phylogenetic relation of *Xenosporium* to that of *Thaxteriellopsis* is yet to be resolved. Morphology of a *Xenosporium* species, *X. ovatum* has some resemblance with young conidia of *Tamhinispora*. However, the conidiophores are well developed and distinct to that of the new genus. In addition, septate pale brown conidial appendages are not seen in *Xenosporium*, which is mainly characterized based on the presence of zero to muriform septate secondary conidia.

Similar to that of *Xenosporium*, *Monodictys*-like anamorphs were also reported in the *Tubeufiaceae* (Seifert et al. 2011). This lineage is also yet to be resolved through a detailed exploration and phylogenetic analysis. Hyde et al. (2010), Vasilyeva & Stephenson (2010) and Boonmee et al. (2011) emphasized the importance of recollection of generic types and epitypification using molecular phylogenetic data so as to progress further to define the generic boundaries in the family *Tubeufiaceae*.
Fig 21 – Phylogenetic tree based on aligned ITS sequences of *Tamhinispora indica* and its closely allied species in *Tubeufiaceae* was performed based on the recent generic concepts of the *Tubeufiaceae* established by Boonmee et al. (2011) using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.77996294 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 268 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

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