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Article

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New records of *Hypoxylon hypomiltum* and *Hypomontagnella monticulosa* from Pilikuththuwa lowland wet zone forest, Sri Lanka

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Abstract

Species of *Hypoxylon* exhibit their highest diversity in the tropics. During an assessment of diversity of hypoxylaceous fungi in the lowland wet zone forest area in Sri Lanka, two new records were reported. Morphological identification of the collected samples was carried out using both macroscopic and microscopic characteristics. Genomic DNA was extracted using modified CTAB method and amplification of both ITS and β-tubulin genes were performed using ITS1 and ITS4 and T1 and T22 primers respectively. In the present study, recognition of species in *Hypoxylon* was carried out using a combined phylogenic analysis of ITS and β-tubulin molecular markers. Phylogenetic tree resulted from combined analysis comprised nine clades. According to the results, two new country records of *Hypomontagnella monticulosa* and *Hypoxylon hypomiltum* were recognized from Pilikuththuwa lowland wet zone forest.

Key words – Hypoxylaceae – Morphology – Phylogeny – Taxonomy

Introduction

Genus *Hypoxylon* (Bull.) was traditionally described in the family Xylariaceae (Ju & Rogers 1996, Stadler 2011). The classical morphological concept of Ju & Rogers (1996) was based on four main characters to distinguish *Hypoxylon* from other genera of Xylariaceae as (1) nodulisporium-like anamorphs, (2) unipartite stromata, (3) solid and homogeneous stromatal tissue below the perithecial layer and (4) stromata not upright. According to Ju & Rogers (1996), the *Hypoxylon* genus was divided in to two sections, section *Hypoxylon* and *Annulata*. Later Hsieh et al. (2005) upgraded the *Hypoxylon* section *Annulata* into a genus level *Annulohypoxylon*, based on a multigene phylogeny and there on, *Hypoxylon* comprises only the species which were categorized under *Hypoxylon* section *Hypoxylon*.

With the current knowledge in taxonomy, phylogeny and chemical profiles, *Hypoxylon* was transferred to the family *Hypoxylaceae* (Ascomycota, Xylariales) with other related genera based on a multigene analysis and morphology of their asexual morphs (Daranagama et al. 2018, Wendt et al. 2018). Due to the simple stromatal anatomy, and the multi-DNA locus genealogy, the taxonomists identified that the species related to *Hypoxylon monticulosum* Mont. as an ancestral clade (Lambert et al. 2019). Lambert et al. (2019) accommodated a new genus *Hypomontagnella* following morphology and chemotaxonomic studies along with multi gene phylogeny. With this addition *Hypomontagnella* now comprised five species (Lambert et al. 2019).

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Target regions of the ribosomal DNA genes subjected to amplification and sequencing have become a promising tool in precise identification and characterization of fungi (Bitzer et al. 2008, Triebel et al. 2005). RNA coding genes such as the nuclear small and large subunit (nucSSU, nucLSU), the mitochondrial small subunit (mitSSU) and the internal transcribed spacer region (ITS) are routinely used in molecular phylogenetic studies of fungi (Lutzoni et al. 2004). However, due to the slow evolution of protein-coding genes such as RNA polymerases (e.g. RPB1, RPB2) and β-tubulin, they are more effective in inferring distant phylogenetic relationships (Hongsanan et al. 2017, Daranagama et al. 2018, Wendt et al. 2018).

Research at national level on taxonomy and phylogeny of *Hypoxylon* is scarce in Sri Lanka. However, few studies have carried out within Sri Lanka to characterize species of *Hypoxylon* (Kuhnert et al. 2014). Pilikuththuwa lowland wet zone forest, where the samples were collected, is a cave forest, which is located just 30 miles from Colombo, in Gampaha district. It covers approximately 200 acres and has approximately hundred small caves spread over. The vegetation of the forest includes the characters of lowland wet zone.

Present study made a major contribution towards the nomenclature of Sri Lankan hypoxylaceous fungal species while producing a stable classification for species based on polyphasic approach. Two new country records of *Hypoxylon hypomiltum* and *Hypomontagnella monticulosa* were found from Pilikuththuwa lowland wet zone forest and presented with illustrations, descriptions and phylogenetic analysis.

Materials & Methods

Specimen collection, examination and isolation

Fresh specimens were collected from Pilikuththuwa lowland wet zone forest from decaying wood materials lying on the ground, during May-July 2018, based on external macro morphological features. Collected samples were placed in paper bags and they were brought to the laboratory for further observations. Both macroscopic and microscopic characters were observed in all collected specimens. Collected hypoxylaceous fungal species were morphologically characterized following the monograph published by Ju & Rogers (1996). Surface colour, colony colour and KOH extractable pigments were recorded according to Rayner's colour chart (1970). Ascomata of collected *Hypoxylon* species were observed using the stereo microscope (Olympus SZ61 model, Philippines). Microscopic characters including asci and ascospores were observed using Phase Contrast Microscope (Olympus CX41 model, Tokyo, Japan). The ascus apical ring was stained using Melzer's reagent. Microphotography was carried out using Olympus DP 26 Mega Pixel camera fitted to the Phase Contrast Microscope (X400 magnification) (Olympus CX41 model, Tokyo, Japan). Measurements of stromata (n=10), perithecia (n=10), asci (n=20) and ascospore (n=20) were made from material mounted in water and the mean values were used in the description. Measurements were made with the Tarosoft (R) Image Frame Work Program and images used for figures were processed with Adobe Photoshop CC version 18.0 (Adobe Systems Inc.), The United States). In order to isolate fungi, the upper surface of any fruiting bodies were excised using sterilized scalpel blade under aseptic conditions. Pure cultures were obtained either from single spore or multispore isolation following the methods detailed by Chomnunti et al. (2014) and Daranagama et al. (2015). The cultures were maintained at 27 °C in MEA in the laboratory. Cultures and herbarium specimens of collected materials were deposited in Department of Botany, University of Kelaniya (UKBC and UKBH).

DNA extraction, PCR and Sequencing

DNA was extracted from cultures grown on malt extracted agar (MEA) plates. The total genomic DNA was extracted from 0.05 to 0.10 g of growing mycelium using modified CTAB protocol following Daranagama et al. (2015). Precipitated DNA was recovered by centrifugation at 12000 rpm for 10 min followed by three steps of purification with 70% ethanol. The air dried,

precipitate was dissolved in 25 µl of nuclease free water and stored at −20 °C until use for amplification reactions.

PCR amplifications were performed for ITS and β-tubulin genes using ITS 1 and ITS 4 (White et al. 1990) and T1 and T22 primer pairs (O'Donnell & Cigelnik 1997) respectively. The total volume of 25 µL reaction mixture [5 µL PCR buffer (1X), 1.5 µL $MgCl_2$ (1.5 mM), 0.5 µL dNTP (0.20 mM), 01 µL of each primer (0.4 µM), 0.25 µL Taq polymerase (1 U/1 µL) and 2.0 µL template DNA (1:10 diluted)] was used for PCR with adjustments of components' volumes and concentration when needed. PCR was carried out in a thermal cycler (Veriti 96-Well Thermal Cycler, ABI Biosystems, USA). PCR conditions were summarized in Table 1. The amplified PCR products were visualized on 1% agarose gel in 1X TBE containing ethidium bromide, using gel documentation system (QUANTUM ST5, Germany) against 100 bp and 1 kb DNA molecular weight markers (Promega, USA).

The PCR products were purified and DNA sequencing was performed at Genetech Pvt Ltd, Sri Lanka and Macrogen Inc. Seoul, Republic of Korea using the same primer pairs.

Table 1 PCR conditions used in PCR amplification.

Gene	PCR primers (F/R)	PCR protocol
ITS	ITS-1/ITS-4	95 ^{o}C: 5 min, (95 ^{o}C: 30 sec, 54 ^{o}C: 30 sec, 72 ^{o}C: 1.30 min, 37 cycles), 72 ^{o}C: 10 min
β-tubulin	T1/T22	95 ^{o}C: 5 min, (95 ^{o}C: 1 min, 54 ^{o}C: 1.50 min, 72 ^{o}C: 2 min, 35 cycles), 72 ^{o}C: 10 min

Sequencing alignment and phylogenetic analysis

Raw sequences were assembled using Bioedit 7.1.3.0. Sequence homologies for the assembled consensus sequences were analyzed using the BLASTn of the NCBI for the rough identification of fresh isolates used in the analysis. Representatives of *Hypoxylon* species downloaded from GenBank were used to infer phylogenetic relationships, including newly generated sequences from 2 strains using both ITS and β-tubulin. Sequences from 47 reliable strains were downloaded from GenBank (Table 2). *Daldinia concentrica* (CBS 113277) was used as the outgroup taxon.

Phylogenetic analysis was performed using a combined alignment of ITS and β- tubulin gene sequences. The consensus sequences were then initially aligned using Clustal X v1.83 and optimized in Bioedit 7.1.3.0 where necessary.

The phylogenetic relationships were analyzed using Maximum likelihood (ML) analysis. ML gene-trees were estimated using RAxML-HPC2 on XSEDE tool (Stamatakis 2006, Stamatakis et al. 2008) available in the CIPRES Science Gateway platform (Miller et al. 2010). For the concatenated dataset all free modal parameters were estimated by RAxML with ML estimate of 25 per site rate categories. The RAxML software accommodated the GTRGAMMA model of nucleotide substitution with the additional options of modeling rate heterogeneity (Γ) and proportion invariable sites (I). The resulting trees were viewed using Figtree v1.4.3. Bootstrap support (BS) values of ML analysis are indicated below or above the branches.

Results

Phylogenetic analysis

The combined phylogenetic dataset of ITS and β-tubulin contains 49 taxa with *Daldinia concentrica* as the out group taxon. The best scoring RAxML tree is shown in Fig. 3. According to the phylogram inferred from combining ITS and β-tubulin sequences (Fig. 3), *Hypoxylon hypomiltum* (UKBC026) from our collection clustered with *H. hypomiltum* (MUCL51845) with bootstrap support of 100% within subclade E. Further, subclade E comprised species of *H. cercidicola, H. petriniae, H. carneum, H. perforatum, H. musceum, H. ochraceum, H. pilgerianum,*

H. samuelsii, and *H. rubiginosum*. *Hypomontagnella monticulosa* collected in the present study was clustered with the type species of *Hypomontagnella monticulosa* (MUCL54604) with 100% bootstrap support within subclade G. Along with *Hypomontagnella monticulosa*, subclade G comprised species of *Hypomontagnella submonticulosa* and *Hypomontagnella barbarensis* as a separate clade. *H. haematostroma, H. fragiforme, H. howeanum, H. ticinense, H. subgilvum and H. rickii* clustered within clade C with high bootstrap supports. Clade D comprised of species of *H. erythrostroma, H. lenormandii, H. fendleri, H. rutilum* and *H. crocopeplum*. *H. trugodes, H. fuscopurpureum, H. aeruginosum, H. begae, H. vinosopulvinatum,* and *H. griseobrunneum* clustered within clade H where *H. pulicicidum, H. investiens* and *H. lateripigmentum* clustered in clade I with high bootstrap support.

Taxonomy

Hypomontagnella monticulosa (Mont.) Sir, L. Wendt & C. Lambert, Mycological Progress 18 (1-2): 190 (2018) Fig. 1

Index Fungorum number: IF 165810; Faces of fungi number: FoF 06136

Saprobic on decaying dicot branches. *Stromata* superficial, effused-pulvinate, forming compact mass, surface brown to black, conspicuous, KOH extractable pigments Vinaceous purple (101). *Perithecia* 211.23 (± 14.99) × 184.10 (±7.74) µm, spherical to hemispherical, *Asci* 46.56 (± 3.99) µm in total length × 1.78 (± 0.07) µm broad, cylindrical, 8-spored, spore bearing part 18.03 (± 2.01) µm, stipe 28.54 (± 2.28) µm, with J+, wedged-shaped apical ring bluing in Melzer's reagent. *Ascospores* 3.88 (± 0.11) × 1.84 (± 0.06) µm, light brown to brown, ellipsoidal-inequilateral, broadly rounded ends, uniseriate, unicellular, sigmoid germ slit, nearlt spore – length.

Culture characteristics – Culture on MEA covering 9 cm plate within 45–50 days at 27 ºC, Pale vinaceous (85) to Vinaceous buff (86) mycelia, with dense Pale vinaceous (85) center, reverse Umber (19), Dark brick (60) center with Hasel (88).

Known distribution – Brazil, Colombia, Guyana, French Guiana, Mauritius, Mexico, Paraguay, Philippines, Sri Lanka, Taiwan, USA

Material examined – Sri Lanka, Western Province, Gampaha district, Pilikuththuwa lowland wet zone forest, on decaying dicot wood, on the track to caves, 7º 03' 50.2119" N and 8º 03' 01.1014" E, 29 June 2018, herbarium = UKBH017, Daranagama and Palapathwala, HYXL 017, living culture = UKBC017.

Notes – *Hypomontagnella monticulosa* which was previously accommodated in *Hypoxylon* was recently upgraded into a separate genus (Lambert et al. 2019). Our collection identified as *H. monticulosa* has morphological similarities with the described isotype of *H. monticulosa* in

Lambert et al. (2019) including vinaceous young stromata and KOH-extractable pigments, papillate ostioles, asci with discoid apical ring and ascospores with more or less spore-length sigmoid germ slit. However, the size of ascospores from our collection is comparatively smaller than those previously described where our collection had 4 µm and previous studies had 5 – 7 µm. Three collections of *H. monticulosa* from the same locality were compared further, where the ascospores of all three materials fall within the same range (3 – 5 µm) of the described material UKBH017. *Hypomontagnella monticulosa* identified from Pilikuththuwa forest clustered with its reference strain (*H. monticulosa*, MUCL54604) from French Guiana with 100% bootstrap support. Thus we conclude that the difference of the ascospores measurements could be due to environmental and geographical factors.

Hypoxylon hypomiltum **Mont.,** *Annls Sci. Nat.*, Bot., sér. 2 13: 356 (1840). Fig. 2

Index Fungorum number: IF 158066; Facesoffungi number: FoF 06137

Saprobic on decaying dicot twigs. *Stromata* superficial, sessile, effused-pulvinate, conspicuous, hemispherical, dense, forming compact mass, discoid, surface black, KOH extractable pigments Citrine (13), Oehrecous (44). *Perithecia* 305.61 (± 27.22) × 250.21 (± 5.56) µm, hemispherical to spherical. *Asci* spore bearing part 47.18 (± 0.68) µm, cylindrical, 8-spored, 81.75 (± 0.49) µm in total length × 5.93 (± 0.17) µm broad, stipe 34.57 (± 0.78) µm with J+, discoid.

Ascospores 7.99 (± 0.10) × 3.26 (± 0.20) µm, dark brown, ellipsoidal-equilateral, uniseriate, unicellular, broadly rounded ends, straight germ slit, spore-length.

Table 2 List of taxa used in the phylogenetic study with their strain numbers, origin, GenBank accession numbers and references.

Species	**Strain Number**	**Origin**	**GenBank accession numbers**		**Reference**
			ITS	**β-tubulin**	
Hypoxylon aeruginosum	MUCL 54620	French Guiana	KC968941	KC977305	Kuhnert et al. 2014
Hypoxylon begae	BCRC 34051	Hawaii (USA)	JN660820	AY951704	Kuhnert et al. 2014
Hypoxylon carneum	MUCL 54177	France	KY610400	KX271270	Wendt et al. 2018
Hypoxylon cercidicola	CBS 119009	France	CBS 119009	KC977263	Wendt et al. 2018
Hypoxylon crocopeplum	CBS 119004	France	KC968907	KC977268	Wendt et al. 2018
Hypoxylon erythrostroma	MUCL 53759	Maritinique	KC968910	KC977296	Kuhnert et al. 2014
Hypoxylon fendleri	MUCL 54792	French Guiana	KF234421	KF300547	Wendt et al. 2018
Hypoxylon fragiforme	MUCL 51264	Germany	KC477229	KX271282	Wendt et al. 2018
Hypoxylon fuscopurpureum	BCRC 34067	USA	JN979421	AY951721	Kuhnert et al. 2014
Hypoxylon fuscum	CBS 113049	France	KY610401	KX271271	Wendt et al. 2018
Hypoxylon griseobrunneum	CBS 331.73	India	KY610402	KC977303	Wendt et al. 2018
Hypoxylon haematostroma	MUCL 53301	Martinique	KC968911	KC977291	Wendt et al. 2018
Hypoxylon howeanum	MUCL 47599	Germany	AM749928	KC977277	Wendt et al. 2018
Hypoxylon hypomiltum	MUCL 51845	Guadeloupe	KY610403	KX271249	Wendt et al. 2018
Hypoxylon hypomiltum	**UKBC 026**	**Sri Lanka**	**MK898827**	**MK905762**	**This study**
Hypoxylon investiens	CBS 118183	Malaysia	KC968925	KC977270	Wendt et al. 2018
Hypoxylon lateripigmentum	MUCL 53304	Martinique	KC968933	KC977290	Wendt et al. 2018
Hypoxylon lenormandii	CBS 119003	Ecuador	KC968943	KC977273	Wendt et al. 2018
Hypomontagnella barbarensis	STMA 14081	Argentina	MK131720	MK135893	Lambert et al. 2019
Hypomontagnella monticulosa	MUCL 54604	French Guiana	KY610404	KX271273	Wendt et al. 2018
Hypomontagnella monticulosa	**UKBC 017**	**Sri Lanka**	**MK898826**	**-**	**This study**
Hypomontagnella submonticulosa	CBS 115280	France	KC968923	KC977267	Kuhnert et al. 2014
Hypoxylon musceum	MUCL 53765	Guadeloupe	KC968926	KC977280	Wendt et al. 2018
Hypoxylon nicaraguense	CBS 117739	Burkina Faso	AM749922	KC977272	Kuhnert et al. 2014
Hypoxylon ochraceum	MUCL 54625	Martinique	KC968937	KC977300	Wendt et al. 2018
Hypoxylon papillatum	ATCC 58729	USA	KC968919	KC977258	Wendt et al. 2018
Hypoxylon perforatum	CBS 115281	France	KY610391	KX271250	Wendt et al. 2018
Hypoxylon petriniae	CBS 114746	France	KY610405	KX271274	Wendt et al. 2018
Hypoxylon pilgerianum	STMA 13455	Martinique	KY610412	KY624315	Wendt et al. 2018

Table 2 Continued.

Species	Strain number	Origin	GenBank accession numbers		Reference
			ITS	β-tubulin	
Hypoxylon porphyreum	CBS 119022	France	KC968921	KC977264	Wendt et al. 2018
Hypoxylon pulicicidum	CBS 122622	Martinique	JX183075	JX183072	Wendt et al. 2018
Hypoxylon rickii	MUCL 53309	Martinique	KC968932	KC977288	Wendt et al. 2018
Hypoxylon rubiginosum	MUCL 52887	Germany	KC477232	KY624311	Wendt et al. 2018
Hypoxylon rutilum	BCRC 34092	France	JF908084	AY951752	Kuhnert et al. 2014
Hypoxylon samuelsii	MUCL 51843	Guadeloupe	KC968916	KC977286	Wendt et al. 2018
Hypoxylon subgilvum	BCRC 34094	Hawai (USA)	JQ009314	AY951754	Kuhnert et al. 2014
Hypoxylon ticinense	CBS 115271	France	JQ009317	AY951757	Hsieh et al. 2005
Hypoxylon trugodes	MUCL 54794	Sri Lanka	KF234422	KF300548	Wendt et al. 2018
Hypoxylon ulmophilum	BCRC 34100	Russia	JQ009320	AY951760	Kuhnert et al. 2014
Hypoxylon vinosopulvinatum	BCRC 34101	Taiwan	JQ009321	AY951761	Kuhnert et al. 2014
Hypoxylon vogesiacum	CBS 115273	France	KC968920	KX271275	Wendt et al. 2018
Annulohypoxylon annulatum	CBS 140775	Texas	KY610418	KX376353	Wendt et al. 2018
Annulohypoxylon atroroseum	ATCC 76081	Thailand	AJ390397	DQ840083	Wendt et al. 2018
Annulohypoxylon michelianum	CBS 119993	Spain	KX376320	KX271239	Wendt et al. 2018
Annulohypoxylon moriforme	CBS123579	Martinique	KX376321	KX271261	Wendt et al. 2018
Annulohypoxylon nitens	MFLUCC 12.0823	Thailand	KJ934991	KJ934993	Wendt et al. 2018
Annulohypoxylon stygium	MUCL 54601	French Guiana	KY610409	KX271263	Wendt et al. 2018
Annulohypoxylon truncatum	CBS 140778	Texas	KY610419	KX376352	Wendt et al. 2018
Daldinia concentrica	CBS 113277	Germany	AY616683	KC977274	Triebel et al. 2005, Kuhnert et al. 2014

Culture characteristics – Culture on MEA covering 9 cm plate within 20-22 days at 27 ^{o}C, smooth, soft, Velvety, Pale vinaceous (85), reverse Dark vinaceous (82), Sepia (63), with symmetric edges.

Known distribution – Brazil, Dominican Republic, French Guiana, Mexico, Puerto Rico, Taiwan, USA

Material examined – Sri Lanka, Western Province, Gampaha district, Pilikuththuwa forest, on the track to caves, 7^{o} 03’ 50.2135” N and 8^{o} 03’ 01.1037” E, 20 July 2018, herbarium = UKBH026, Daranagama and Palapathwala, HYXL 026, living culture = UKBC026.

Notes – *Hypoxylon hypomiltum* from our culture collection has similar characters with previously studied species. Both of them have effused-pulvinate stromata, similar lengths in perithecia, more or less similar lengths in asci. Also both of them have equilateral, spore length germ slit in ascospores. The species identified as *Hypoxylon hypomiltum* from Pilikuththuwa forest clustered with its reference strain (*H. hypomiltum*, MUCL52887) from Guadeloupe with 100% bootstrap support.

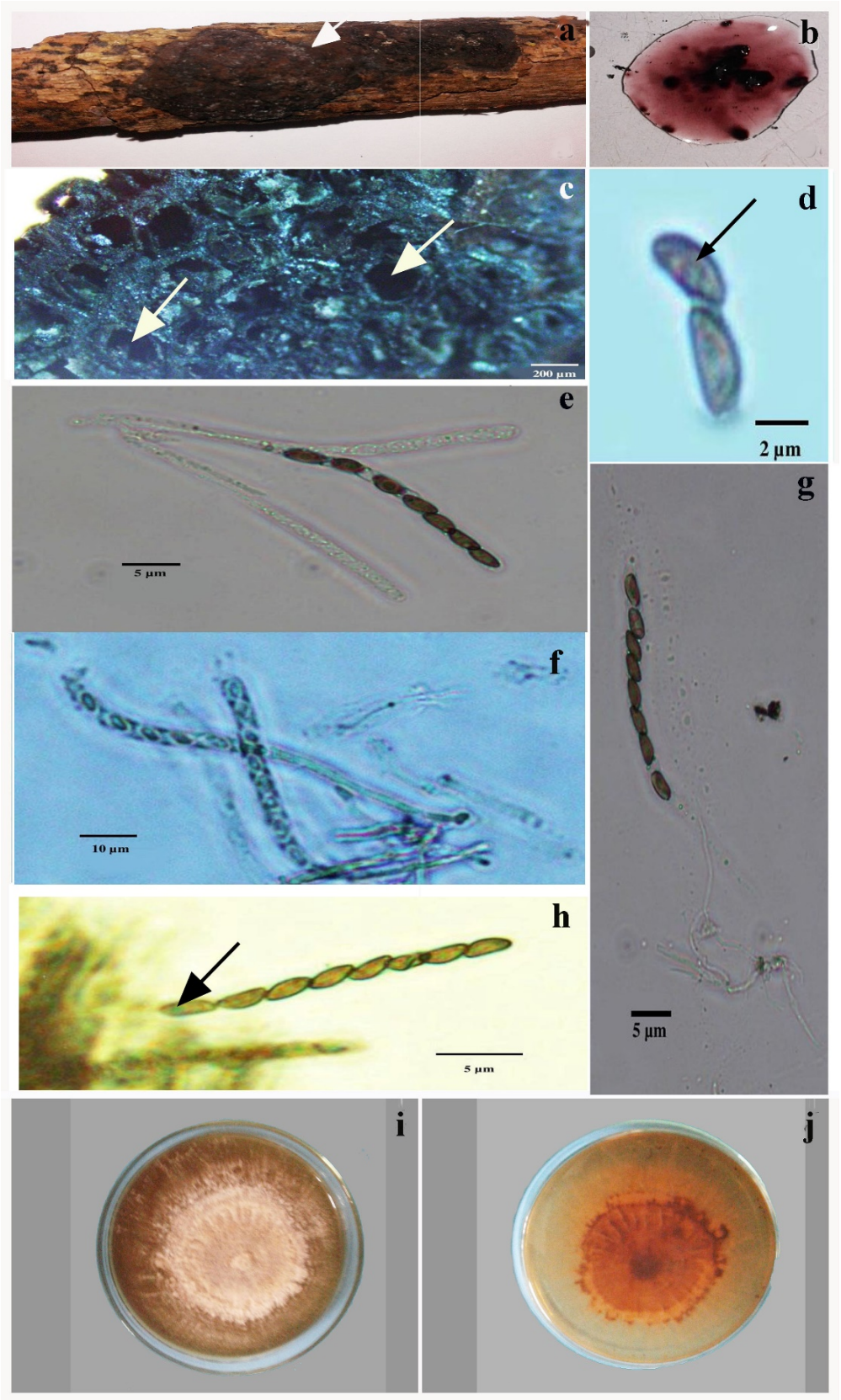

Fig. 1 – Morphological features of *Hypomontagnella monticulosa* (HYXL 017). a Appearance of stromata on substrate (shown by arrow head). b KOH extractable pigments. c Cross section of stromata showing ascomata (shown by arrow heads). d Ascospore (germ slit shown by arrow head). e, f, g Asci (germ slit shown by arrow head). h Asci in Melzer's reagent (J+ apical ring shown by arrow head). i Upper surface of the culture. j Lower surface of the culture (Culture on MEA after 45 days).

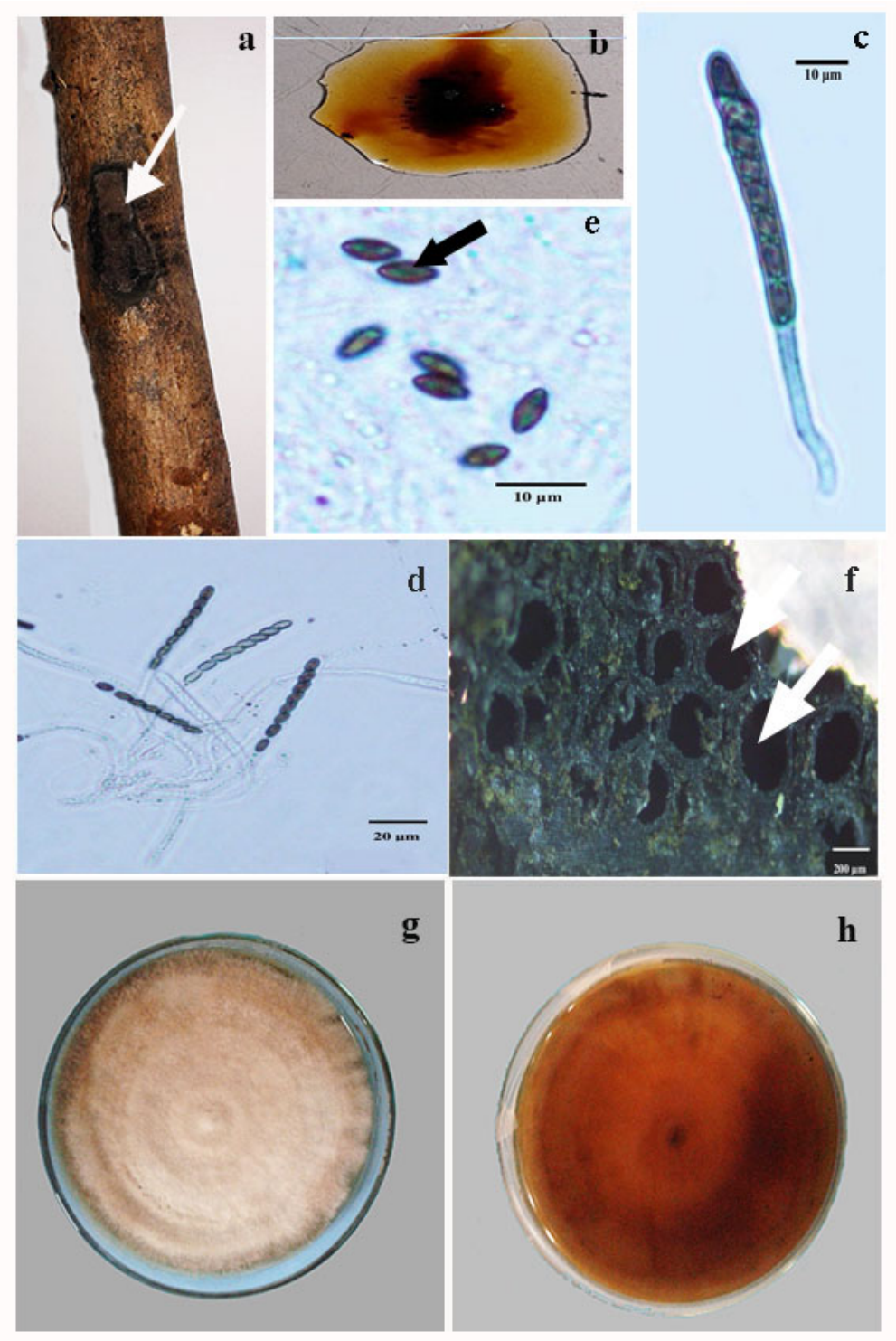

Fig. 2 – Morphological features of *Hypoxylon hypomiltum* (HYXL 026). a Appearance of stromata on substrate (shown by arrow head). b KOH extractable pigments. c, d Asci. e Ascospore (germ slit shown by arrow head). f Cross section of stromata showing ascomata (shown by arrow heads). g Upper surface of the culture. h Lower surface of the culture (Culture on MEA after 20 days).

Discussion

For the preliminary identification and characterization of the collected samples, morphological characters of sexual morph were considered in this present study. Depending on the above features, the identification of the species was carried out using keys provided by Ju & Rogers (1996). As a result, two species, *Hypomontagnella monticulosa* and *Hypoxylon hypomiltum* were recorded from Pilikuththuwa lowland wet zone forest.

Application of RNA coding genes and ITS was used for molecular phylogeny of fungi (Lutzoni et al. 2004). But in very recent past, number of studies (Daranagama et al. 2018, Wendt et al. 2018) used protein coding genes such as α-actin and β-tubulin, because the usage of protein coding genes proved to be more efficient in phylogenetic studies.

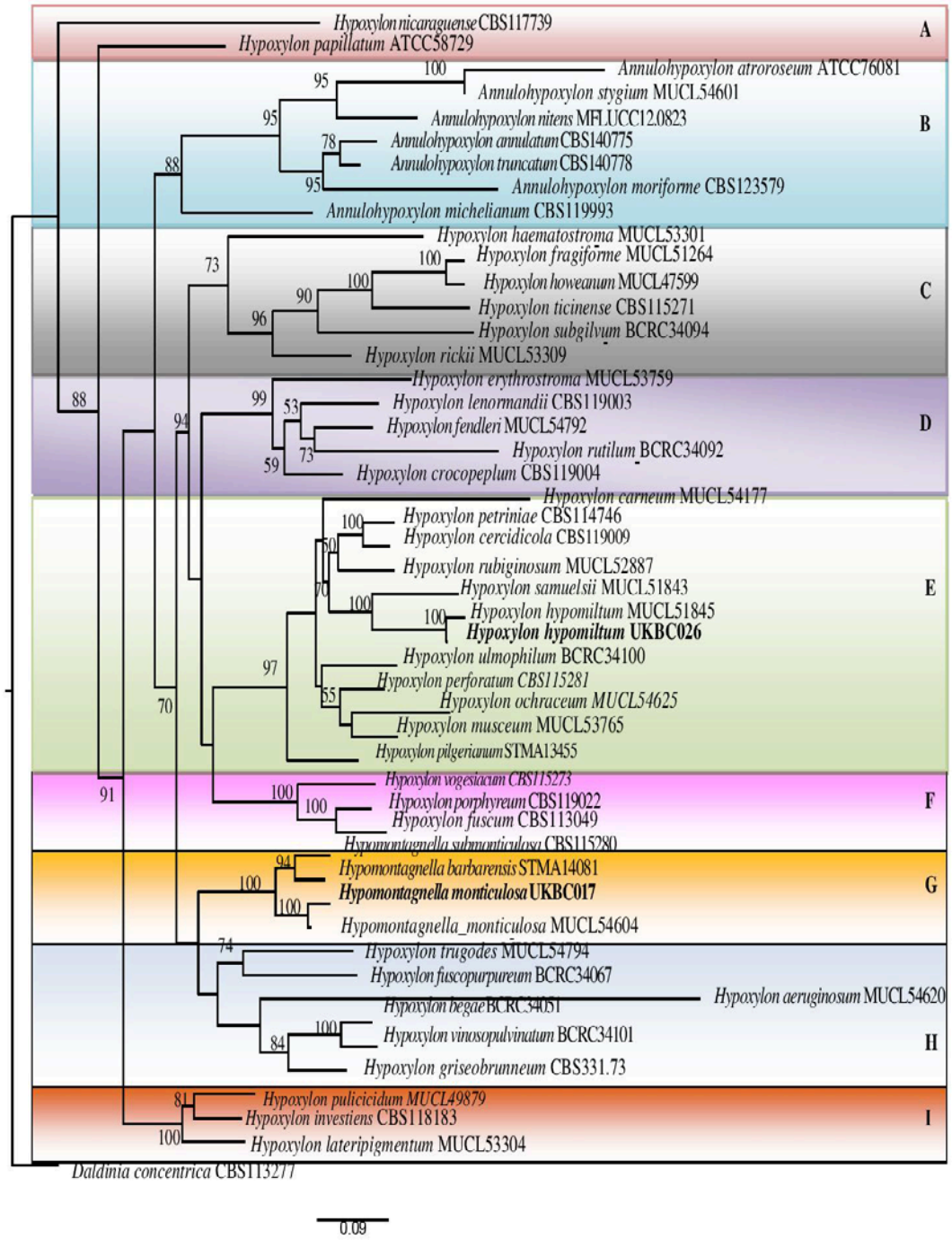

Fig. 3 – Phylogram constructed with RAxML using combined dataset of ITS and β-tubulin sequences. Bootstrap support values ≥50% are indicated below or above the nodes. *Daldinia concentrica* was used as the out group taxon. Sequences obtained from current study are shown in bold.

Single gene tree of ITS did not provide a good resolution of terminal taxa. Even though, single gene tree of β-tubulin had a low resolution within the species, it was more informative than single gene tree of ITS. Due to the poor resolution of both single gene trees, the combined gene tree

of ITS and β-tubulin was used. As expected, the combined analysis was more successful and it provided better resolution within species. Thus, the combined analysis was more informative and successfully used in phylogenetic studies to infer the phylogenetic relationship among species.

The combined phylogenetic tree comprised nine clades including *Annulohypoxylon* as a separate clade – clade B with all *Annulohypoxylon* strains.

Sri Lanka, though a small country, has a great deal of biodiversity of fungi. However, information available on Sri Lankan fungi is scattered. The studies on fungal diversity were initiated before 1800s in Sri Lanka, and the first Sri Lankan fungi to be recorded were *Peziza ceylonische* and *P. lembosa* (Karunarathna et al. 2012). From there, number of mycologists carried out several studies on Sri Lankan fungi (Karunarathna et al. 2012). According to Karunarathna et al. (2012), current statistics suggest that there could be as many as 25,000 species, of which only a little more than 2,000 are presently known.

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