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First documentation of*Termitomyces srilankensis* from Kerala, India

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A new species of termite mushrooom, *Termitomyces srilankensis* was documented for the first time in India based on specimens collected from Thrissur and Wayanad districts of Kerala. This species is morphologically characterized with medium to large sizedplano-convex pileus with whitish brown to deep brown colour, spiny form to conical formed light brown coloured perforatorium with white coloured compactly arranged gills.Stipe, long, slender, cylindrical and solid (6.6-14.6 x 2.3-10.6 cm) with a grayish coloured pseudorhiza (6.8-11.8x 2.4-4.2 cm). Annulus was absent. Microscopic examination of morphological structures, such as the pileipellis layer, basidia, cystidia, and basidiospores, provided further taxonomic insights into the species. Confirmation on the identity of the new species was carried out by molecular characterization by sequence homology of Internally Transcribed Spacer (ITS) and Large subunit (LSU) regions. Further, nnutritional profile of *T. srilankensis* was analyzed, revealing significant levels of carbohydrates, protein, crude fibre, and vitamin C, with a high moisture content. The discovery of this species expands the known diversity of *Termitomyces* in the Indian subcontinent and underscores the region's mycological richness.

Key words: Diversity, homology, molecular characterization, morphological characterization, mushroom

INTRODUCTION

Edible mushrooms thrive in connection with the woody components of trees or in soil manifesting as parasites, saprophytes, or symbiotic entities. These mushrooms play diverse ecological roles in nature and are extensively utilized by humans for both culinary and medicinal purposes.

Among various mushroom types, those associated with termites are frequently favoured and highly esteemed in numerous countries due to their flavour, aroma, visual appeal, medicinal properties, and suitability as alternatives to meat or fish.

Termite mushrooms, belonging to the genus *Termitomyces* under the family Lyophyllaceae (Moncalvo *et al*. 2000), are cultivated by termites of the subfamily Macrotermitinae. The mutualistic relationship between *Termitomyces* fungi and termites originated at least 31 million years ago (Nobre *et al*., 2011), where, termites create a stable environment for fungal growth and aid in spore dispersal, while *Termitomyces* serves as a nutritional resource for the termites.

The presence of *Termitomyces* in termite nests was first documented by the German scientist König (1779) at Tanjore area of South India andthe genus *Termitomyces* was formally established by Heim (1942). To date, more than 30 species of this genus are known and were accepted in the 10th edition of the Dictionary of the Fungi (Frøslev *et al*. 2003; Kirk *et al*. 2008). *Termitomyces* is frequently found in the ecosystems of tropical and sub-tropical regions of the world and most of the type species are discovered from Africa followed by south-east Asia. These mushrooms exhibit significant

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diversity across various regions of India, with a total of 22 species reported from different parts of the country (Atri *et al*.2005). The regional distribution of *Termitomyces* species in India shows distinct concentrations. The majority of the inventory on *Termitomyces* has been conducted in Kerala (Farook *et al*. 2013), where 15 species have been documented, Goa follows with 10 species, and Karnataka with 9 species. In contrast, Tamil Nadu and Maharashtra have recorded 2 and 3 species, respectively.These regional variations reflect the diverse ecological niches and habitats that support the growth of *Termitomyces* species across India.

The identification of termite mushroom is performed using macro and micro-morphological and molecular characteristics. Phenotypic traits include the shape, size and color of the fruiting body, nature of perforatorium and the presence or absence of annulus, pseudorhiza and fissile margins of maturepileus and the micro-morphological characters include the nature of pileipellis, basidia, sterile structures of hymenial layer and basidiospores.Characterization based on molecular methods offers very precise and reliable means for species level identification and techniques such as analysis of the 18S rRNA or internal transcribed spacer (ITS) regions, can be used to identify mushrooms at any developmental stage(Rajarathnam and Thiagarajan, 2012).Ediriweera *et al*. (2023) were the first to document *Termitomyces srilankensis* from SabaragamuwaProvince, Kegalle District, Sri Lanka. The identification was based on the amplification of the nuclear ribosomal internal transcribed spacer (nrITS) and large subunit (nrLSU) regions, using the primer pairs ITS5/ITS4 and LROR/LR5.

Between June 2023 to October 2024, the occurrence of *Termitomyces* was observed in the central and northern regions of Kerala where, purposive sampling survey was conducted for documentation of termite mushrooms followed by studies to identify different species based on morphological characteristics and by analyzing the homology of the internal transcribed spacer (ITS) and LSU regions. The study revealed the presence of *T. srilankensis* at four locations of Thrissur and Wayanad districts of Kerala occurring during South-West and North East monsoon seasons in humus rich soils under high humid conditions.

MATERIALS AND METHODS

Survey for the collection of Termitomyces spp.

Purposive sampling survey was conducted in Central and Northern parts of Kerala during the South-West and North-East monsoon seasons from June 2023 to October 2024. The survey covered different agro-ecological units (2, 6, 7, 10, 11, 20, and 21) of Thrissur, Wayanad and Kasaragod districts of Kerala.

Morphological characterization

The collected samples were morphologically characterized based upon the size, shape and colour of pileus, perforatorium, lamellae, and stipe, and presence or absence of annulus and pseudorhiza as per the data sheet provided by Nair and Devi (1986). Micromorphological characters were recorded using fresh and mature sporocarps of *Termitomyces* spp. and thin sections of the gills were taken to observe the shape and size of basidia, cheilocystidia, pleurocystidia and basidiospores and were stained with 1 per cent lactophenol cotton blue stain for micro-morphometric observations. The dimensions of basidia, cystidia, basidiospores and type of pileipellislayerwere measured using compound microscope (Leica- ICC50 HD, USA) at 100 X magnification.

Molecular characterization

The collected samples were identified up to genus level based on the observable morphological characters. The species level identification was carried out using molecular techniques which included fungal DNA isolation, amplification of the DNA template followed gel electrophoresis, sequencingof the nuclear ribosomal internal transcribed spacer (nrITS) and large subunit (nrLSU) regions and *in silico* analysis. The DNA from the fresh mushroom fruiting body was isolated using 2% CTAB buffer method; here 300 mg of sample were grinded with 200 ml of CTAB

buffer and transferred into 2 ml eppendorf tube and incubated at 65°C for 30 minutes, centrifuged at 10,000 rpm for 10 minutes. Supernatant was transferred into sterile eppendorf tube, added with equal amount of Chloroform: isoamyl alcohol (24:1) and spinned at 10,000 rpm for 10 minutes. The spinned tube contain three layers, from that upper aqueous phase with clear solution were taken into a clean microfuge tube and added with 0.6 volume of ice cold isopropanol to precipitate the DNA and stored at -20°C for overnight. Thenprecipitate is isolated by spinning the tube at 10,000 rpm for 7 minutes and the pellet was washed with 70% ethanol. The pellet containing DNA was suspended in 50 µl TE buffer and maintained at -20°Cfor future use.

The isolated DNA was used for PCR amplification with Thermal Cycler. Where, 30 reactions were followed with a PCR mix of total 20 ìl. PCR mix includes 1 µl of DNA template, 0.6 µl of each ITS1F (5' TCCGTAGGTGAACCTGCGG 3')and ITS4R (5'TCCTCCGCTTATTGATATGC 3'), 10 ìl of 2X PCR master mix and 7.8 µl of double distilled water. The large subunit (nrLSU) regions were amplified using the primers LROR (ACCCGCTGAACTTAAGC) and LR7 (TACTACCACCAAGATCT).The amplification was carried out at different steps. Initial denaturation at 95°C for 2 minutes, denaturation at 95°C for 45 seconds, annealing at 54.6°C for 30 seconds, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes.

The amplified DNA was run on 2% agar gel, at 70V for 45 minutes along with the 5 µl tracking dye Ethidium bromide. Bands were observed under UV light and bands thickness was documented using Gel Doc. The PCR product was sent for sequencing to the Genespec Lab at Kochi. Each sample ITS forward and reverse sequences were combined using *insilico* cap3 conting assembly software. Utilizing BLASTn from the NCBI database, the homology of the retrieved sequences was determined.

Proximate analysis of Termitomyces spp.

The proximate analysis of *Termitomyces* spp. was done for carbohydrates, protein, crude fibre, vitamin C, ash content and moisture percentage. The specimens were dried in the hot air oven and powdered. The moisture content was estimated on wet weight basis whereas the other parameters were analyzed on dry weight basis following standard methods of analysis.

Estimation of Carbohydrates

The carbohydrate content was determined by Anthrone method (Hedge and Hofreiter, 1962), 100 mg of the powdered sample was hydrolyzed with 5 ml of 2.5N hydrochloric acid by heating it in a boiling water bath for 3 hours. Neutralization was carried out by adding solid sodium carbonate until the effervescence ceased.The total volume was then adjusted to 100 ml with distilled water, followed by centrifugation. The supernatant was collected, and a 1 ml aliquot was taken for further analysis. Different concentrations of glucose was used as standard and 4 ml of Anthrone reagent was added to record the colour change from yellow to different shades of green, absorbance of both sample and glucose standards was taken at 630 nm in Spectrophotomer. The OD values of glucose standards were used to construct the standard graph and from standard graph, concentration of the carbohydrate were expressed in g/100g of dry weight (DW).

Amount of carbohydrate/ 100 mg of sample =

$$\frac{\text{mg of glucose} \times 100}{\text{Volume of test sample}}$$

Estimation of protein

The Bradford (1976) protein assay is a simple, fast, and cost-effective method to measure protein concentration by binding proteins to the dye Coomassie Brilliant Blue G-250. In this assay, protein samples are prepared in Bovine Serum Albumin (BSA), and 5 ml of diluted dye is added to each sample. After mixing, the colour is allowed to develop for 5 to 30 minutes, and the absorbance is read at 595 nm. A standard curve is then used to calculate the protein concentration in the samples based on their absorbance.

Estimation of crude fibre

Crude fibre per cent was estimated by following the procedure of James (1995). A 0.5 g of sample

was digested with 12.5 ml of 10% v/v nitric acid and 12.5 ml of 2.5% v/v sodium hydroxide solutionand the mixture was boiled with constant stirring for 30 minutes each time with acid and alkali solutions separately. The residue was strained and washed, and transferred to a clean, dry crucible or petri dish.After drying in an oven, the weight of the residue was measured. The percentage of crude fibre was calculated using the following formula.

$$\text{Percentage weight of crude fibre} = \frac{\text{Weight of crude fibre}}{\text{Initial weight of sample taken}} \times 100$$

Estimation of vitamin C

Vitamin C content was estimated by following the procedure of Harries and Ray (1935). 10 g of powdered dry mushroom was diluted with 4 % of 100 ml of oxalic acid. Again the solution was Dilutedto ten times before titration (1:10). 5 ml of this diluted solution was used for titration with 2,6-dichlorophenol indophenols (DCIP)dye solution, repeating the process three times to obtain an average value. Then, 5 ml of the vitamin C working solution was transferred to a 100 ml conical flask, containing 10 ml of oxalic acid, and titrated against DCIP solution until a pale pink color appears, indicating the endpoint. Repeat the procedure three times to obtain an average value Ascorbic acid concentration (mg/100ml) = (0.5mg/ml x V2 ml x 100ml) / (V1 ml x weight of sample (g)); where V1 is the volume of dye consumed for a standard ascorbic acid solution and V2 is the volume of dye consumed for the sample being tested.

Estimation of water content

Five grams of the specimen (W1) was taken in a pre-weighed crucible and dried in hot air oven until a constant weight was obtained (W2). The difference between the initial (W1) and final (W2) weight gives the moisture content, which is then converted and expressed in percentage.

$$\text{Per cent moisture content} = \frac{W1 - W2}{W1} \times 100$$

Estimation of ash content

3 g of the specimen was placed into a crucible and subjected to heat in a moisture extraction oven at 550°C until it transformed to a white color, indicating the absence of carbon. Afterwards, taken out from the oven, and allowed to cool in a desiccator until it reached the room temperature, and then used for reweighing. The mass of the remaining ash was then determined as the ash content.

$$\text{Percentage (\%) Ash} = \frac{\text{Weight of Ash}}{\text{Weight of original sample}} \times 100$$

RESULTS AND DISCUSSION

A mushroom survey was conducted during south west and north east monsoon seasons of 2023 – 2024 to document different *Termitomyces* species ofCentral and Northern parts of Kerala. Among the 28 samples collected, four samples observed from different Agroecological Units (AEU's) of Thrissur (AEU 10) and from Wayanad (AEU 20) districts (Table1) showed characters of *T. srilankensis*, a hitherto unreported species from India and the details of macro and micro-morphological and molecular characterization of the samples are presented herewith.

Morphological and molecular characterization

The mushroom fruiting body was observed to be medium to large sized, with convex to plano-convex pileus, whitish brown to brown coloured, 10.2-14.0 cm in diameter with margins initially entire and fissile when mature.Perforatoriumwas spiny form to tiny pointed, or conical to umbonate, white to brown coloured.Lamellae werewhitish, thick, compactly arranged towards the centre and adnexed, 2.8-6.3 cm long and 0.3-0.5 cm wide. Stipe was whitish to creamy white, long, slender, cylindrical, solid, tapering towards distilled portion, with a length of 4.5-14.6 cm and width of 2.3-10.6 cm. Pseudorizha was slender, cylindrical, solid, tapering towards the distal end, creamy white to dark brown coloured, 7.2-12.0 cm long and 1.9-4.2 cm wide. These characteristics of Samples T13, T14, T15 and W4 are depicted in Figs.1-4,A-D.Micro-morphological structures revealed the pileipellis layer to be cutis to ixotrichodermium form.

Basidia, clavate, 7.16-26.62 x 4.42-8.54 μm in size, tetra-sterigmatted, bearing double walled,

Table1: Location particulars and designated codes of the collected samples of *T. srilankensis*

District	AEU	Location	Geographical coordinates (latitude & longitude)	Date of Survey	Designated codes
Thrissur	10	Vellanikkara	Lat ; 10.551739° Long : 284781°	26/06/2024	T13
		Vellanikkara	Lat : 10.548623° Long : 76.285222°	26/06/2024	T14
		Vellanikkara	Lat : 10.548177° Long : 76.285579°	27/06/2024	T15
Wayanad	20	Ambalavayal	Lat : 11.61279° Long :76.213148°	24/08/2024	W4

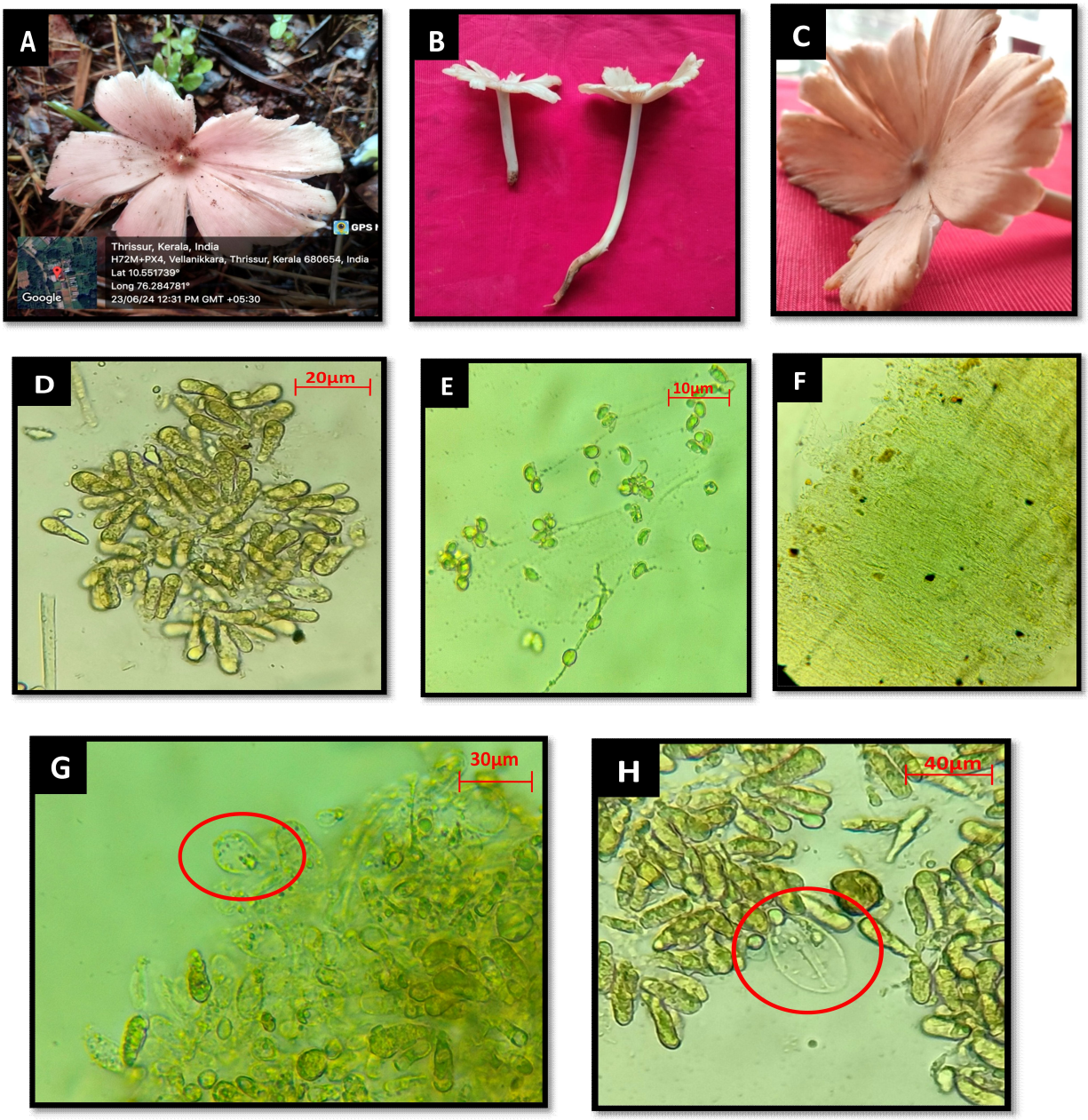

Fig. 1. Habitat & location (A), Fruiting body (B), Perforatorium (C), Basidia (D), Basidiospores (E), Piliepellis layer (F), Pleurocystidia (G), Cheilocystidia (H) of T13 sample.

Table2: GenBank Accession numbers of deposited ITS sequences *Termitomycessrilankensis*

Sl. No.	Sample name	Designated code	Submitted id	Accession number for ITS region
1	T13	TVV13	SUB14812987 seq1	PQ505135
2	T14	TVV14	SUB14814852 seq2	PQ510371
3	T15	TVV15	SUB14814935 seq3	PQ510376
4	W4	WAA4	SUB14814992 seq4	PQ510379

Table3: Proximate analysis of *T.srilankensis* in comparison with *T. microcarpus* on dry weight basis (g/100g)

Species	Carbohydrate (g/100g) DW	Protein (g/100g) DW	Crude fibre (%)	Vitamin C (mg/100g) DW	Moisture content (%)	Ash content (%)
T. srilankensis	34.92	17.66	8.1	30.64	91.72	10.38
T. microcarpus	46.9	14.4	6.6	26.40	87.14	7.75

ellipsoid, hyaline basidiospores measuring4.31-7.49 x4.13-6.44ìm in size. Pleurocystidia were pyriform to clavate form, thin walled, hyaline structures with a measurement of 13.0-29 x 10.89-21.35 ìm. Cheilocystidia were ovoid to clavate, thin walled, hyaline sterile structures with 29.94-33.31 x 15.07-26.61ìm in size, as shown in Samples T13, T14, T15 and W4 (Figs.1-4,E-I). The macro and micro-morphological characters observed from the study were in line with those detailed by Ediriweera *et al*. (2023).

Ecology, Habit, Habitat and periodicity

Obligatory, solitary, growing in lateriticand forest loam soils of Kerala with high humus content. Generally observed near the mixed vegetation. Observed to occur only once in a rainy season. Out of 28 samples collected, four samples (T13, T14, T15 and W4) showing characters of *T. srilankensis* were taken for species level identification *via* molecular techniques.The forward and reverse nucleotide sequence data obtained by ITS sequencingcombined using *insilico* cap3 conting assembly software was compared with known sequences of nucleotides available in NCBI*via* BLASTn showed that, the sample T13, and showed 98.11 per cent similarity with *Termitomyces srilankensis* with query coverage of 98 per cent with accession ON685313.1. T14 and T15 samples, exhibited 98.78 per cent similarity with *Termitomyces srilankensis* with query coverage of 100 per cent, with the associated accession number ON685313.1 and the contig sequences of the sample W4, from Wayanad district, had shown 99.85 per cent similarity with *Termitomyces srilankensis* having query coverage of 99 per cent with accession number ON685313.1. These results confirmed the identity of all the four samples as *Termitomyces srilankensis*. The sequences were submitted at NCBI database and the corresponding GenBank accession numbers are detailed in Table 2. Ediriweera *et al*. (2023), according to their studies on the morphological characters and phylogenetic analyses based on samples collected from Sabaragamuwa Province, Kegalle District of Srilanka described the new species, *T. srilankensis* establishing its separate identity from the already reported species of *Termitomyces.*

In the present study, for further confirmation of the species, amplification of LSU regions of two samples T 15 and W 4 were carried out using LR7 and LROR primers. The results revealed that T15 sample showed 93.60 per cent similarity with *Termitomyces srilankensis* having query coverage of 50 per cent with accession PP915963.1 whereasthe sample W4 has shown 94.28 per cent for *Termitomyces srilankensis* with

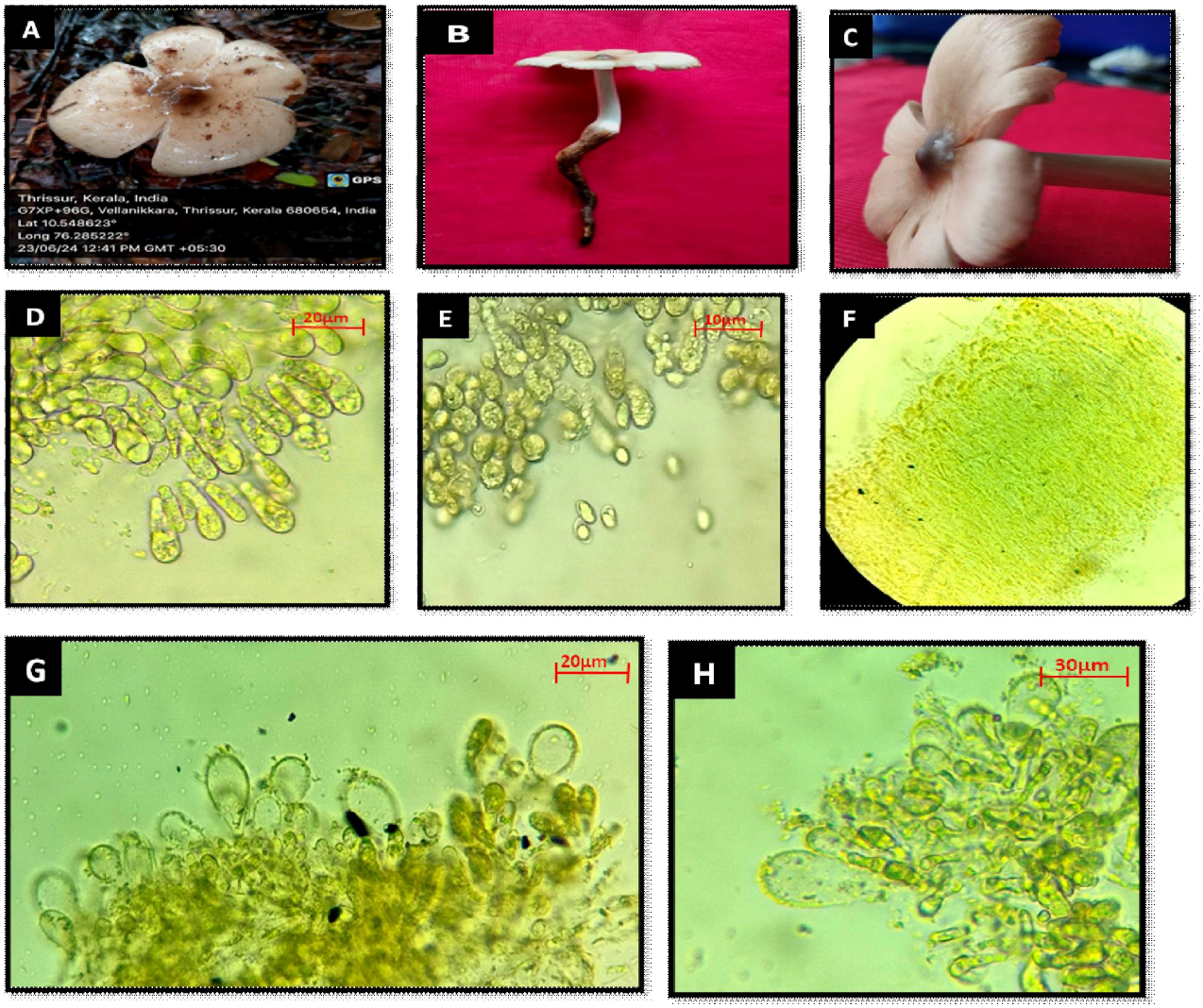

Fig 2. Habitat & location (A), Fruiting body (B), Perforatorium (C), Basidia (D), Basidiospores (E), Piliepellis layer (F), Pleurocystidia (G), Cheilocystidia (H) of T14 sample.

query coverage of 47 per cent having the accession number PP915963.1.

Nutritional composition of Termitomces srilankensis

The different nutritional components like carbohydrates, proteins, crude fibre, vitamin C, ash content and moisture percentage were estimated with respective procedures mentioned earlier and the data was compared with that of *T. microcarpus*, the most commonly occurring species of *Termitomyces* in Kerala.

Carbohydrates and protein contents

Carbohydrates are important components in *Termitomyces* where, *Termitomyces srilankensis* studied in the present study was estimated to contain an average of 34.92g/100g ofdry weight in comparison with *T. microcarpus* having carbohydrate content of 46.9 g/100g (Table3). According to studies by Nabubuya *et al*. (2010),*T. microcarpus* contained higher level of carbohydrates of 48.37%.Kanscie*tal*. (2003) recorded similarly a higher level of carbohydrates in *Termitomyces* with their contents varying from 43.7 g/100 g of *T. letestui* to 57.4 g in *T. schimperi* with average content of49g/100g of DW. The protein content was observed to be an average of 17.66 g/100g DW in contrast to that of *T. microcarpus*, having an estimated protein content of only 14.4 g/100 g. Studies by Kanscie*tal*. (2003) also recorded a higher level of protein in *Termitomyces* with their contents

varying from 14.5 g/100g DW in *T. schimperi* to 19.7 g/100DW in *T. letestui*, with an average of 16.7 ± 1.7 g/100g DW in all the species studied.Gunasekara *et al.* (2021) recorded similar results of higher content of crude protein (28.54-31.05% DW) in *Termitomyces* mushrooms, among all the studied samples.Nabubuya *et al*. (2010) estimated a protein content of 25.5% DW in *Termitomyces microcarpus*.

Vitamin C and crude fibrecontents

The vitamin profile of all the *Termitomyces* mushrooms from the present study were characterized by an appreciable amount of vitamin C (ascorbic acid), varying between 24.90 mg to 36.01 mg/100 g DW. The vitamin C content of *Termitomyces srilankensis* was estimated to contain an average of 34.64 mg/100 g DW whereas in case of *T. microcarpus*, the estimated value was 26.4. Studies by Nakalembe *et al.* (2015) showed that the estimated Vitamin C contents in *Termitomyces* were in moderate amounts (11.05–21.40 mg/100 g), contributing 14.7–28.5% of the recommended FAO/WHO (2005) daily intake of 75 mg/day. LohYoboue *et al*. (2020) reported a vitamin C or ascorbic acid content of the wild edible mushroom *T.letestui* of 20.10 mg / 100g which was slightly lower than the content in *T. srilankensis*.

The nutritional profile of *Termitomyces* mushrooms is further enhanced by their substantial amount of crude fibre content, which has significant implications for gastrointestinal health and satiety. The *Termitomyces srilankensis* from the present study was observed to contain crude fibre content of 8.1 %, which slightly a higher content as compared to that of *T. microcarpus* showing a crude fibre content of 6.6 %. Studies by Nakalembe *et al.* (2015) revealed a crude fibre content of 3.95 % and 3.08 % in *Termitomyces microcarpus* and *Termitomyces tyleranus* respectively, and 7.69 % in *Termitomyces clypeatus*. Gunasekara *et al.* (2021) also reported a higher range of insoluble fibre content of 26.64 ±1.87, 15.28 ± 0.42, 22.72 ± 2.93 g/100g DW in *T. eurrhizus, T. microcarpus*, and *T. heimii* respectively.

Moisture and ash cintents

Mushrooms are known for their high water content, with most varieties consisting of about 90% water. This high moisture level contributes to their low calorie count and makes them a lightweight, nutrient-dense food. According to the our estimation, *Termitomyces srilankensis* contains an average of 91.72 % of water, whereas in case of *T. microcarpus*, the estimated value was 87.14. The per cent of water content from the present study was in line with the report of Gunasekara *et al.* (2021) where they observed 92.08 ± 1.07, 93.36 ± 0.53, and 92.79 ± 0.12 per cent of water content in *T. eurrhizus, T. microcarpus*, and *T. heimii* respectively.

As per the proximate analysis for ash content in *T. srilankensis* samples showed an average ash content of 10.38 % whereas in case of *T. microcarpus* samples, the value was 7.75 %. Results of the studies by Badhai *et al*. (2023) comparing three *Termitomyces* species, revealing variations in their nutritional composition showed that *T. microcarpus* recorded the highest ash content (24.6%) among the different species studied.

Based on the review of existing literature, *Termitomyces srilankensis* has not been documented in Kerala. Consequently, this observation represents the first known record of *Termitomyces srilankensis* from the region, marking a significant addition to the documented fungal biodiversity of Kerala.

CONCLUSION

The current study presents the first documentation of a new species of the termite mushroom, *Termitomces srilankensis* from Thrissur and Wayanad districts of Kerala, India along with its morpho-taxonomy and detailed DNA sequence-based analysis. The identification was based on both macro and micromorphological characteristics of the fresh fruiting body, along with molecular characterization through high-percentage sequence homology of the ITS region and also based on LSU sequences. Proximate analysis revealed that the reported species contains substantial levels of carbohydrates, proteins, vitamin C, and crude fiber.

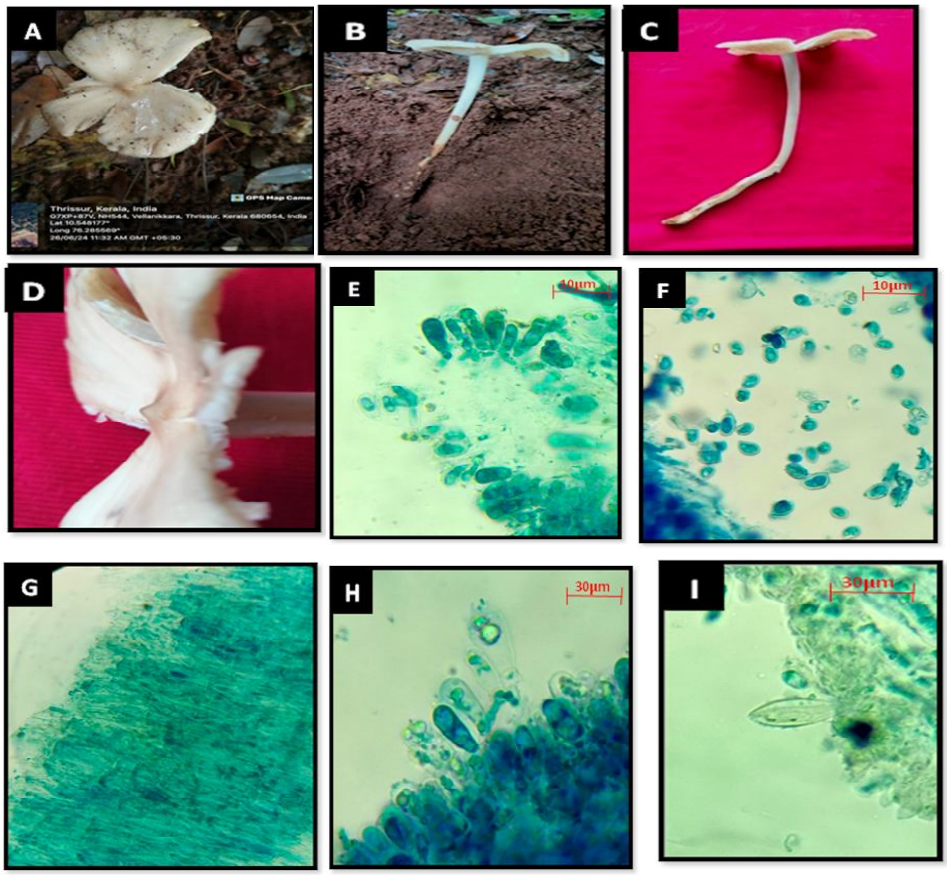

Fig 3. Habitat & location (A), Fruiting body along with soil type (B), Basidiocarp (C), Perforatorium (D), Basidia (E), Basidiospores (F), Piliepellis layer (G), Pleurocystidia (H), Cheilocystidia (I) of T15 sample.

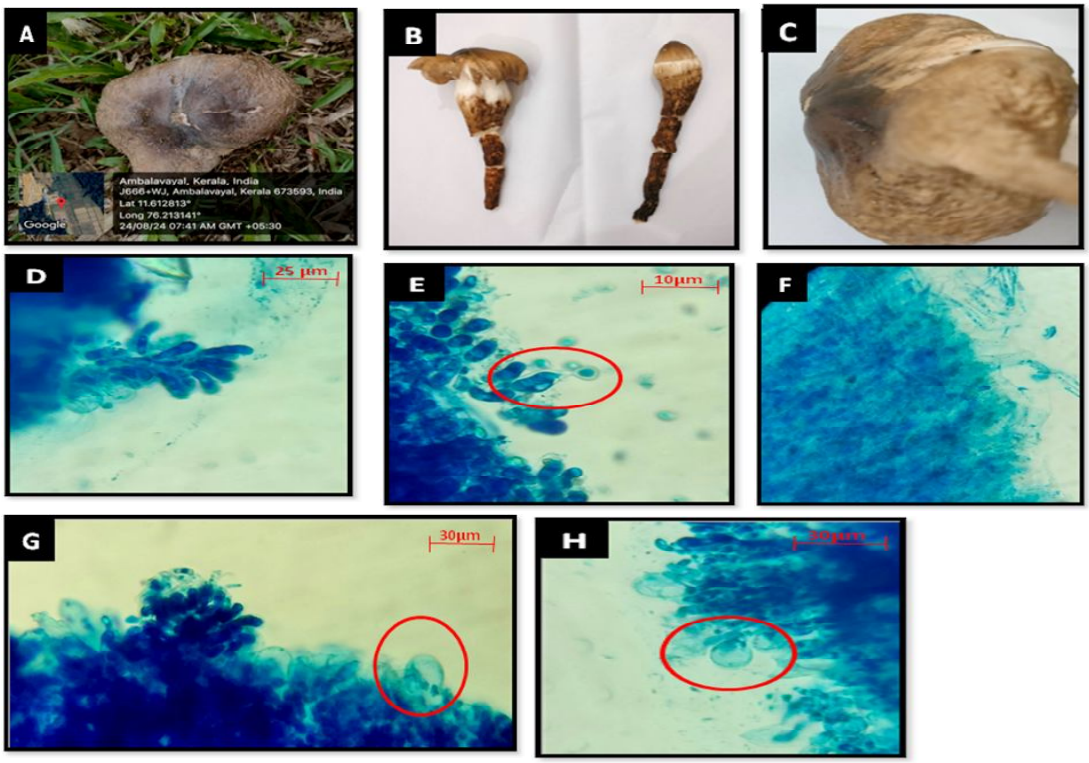

Fig 4. Habitat & location (A), Fruiting body (B), Perforatorium (C), Basidia (D), Basidiospores (E), Piliepellis layer (F), Pleurocystidia (G), Cheilocystidia (H) of W4 sample.

DECLARATION

Conflict of interest: Authors declare no conflict of interest

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