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Short Communication

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SHORT COMMUNICATION

New record of *Tulostoma squamosum* (Agaricales: Basidiomycota) from India based on morphological features and phylogenetic analysis

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Abstract: *Tulostoma squamosum* is reported for the first time from India. A comprehensive macro-morphological description, field photographs along with microscopic observations, and comparisons with morphologically similar and phylogenetically related taxa are provided. Nucleotide sequence comparison and an estimation of evolutionary divergence between *Tulostoma squamosum* sequences across different geographic origin are also provided.

Keywords: Evolutionary divergence, new record, nrDNA ITS, phylogenetic analysis.

The genus name *Tulostoma* was coined by the African mycologist Christiaan Hendrik Persoon in 1801 for the taxa possessing characters like two layered peridium and a woody stalk. The genus is cosmopolitan in distribution comprising of ca. 140 accepted species and mostly found across habitats like sandy soils, forests, pastures, on road sides etc. (Wright 1987; Lima & Baseia 2018).

During repeated field trips by the authors for exploring the hidden macrofungal diversity of West Bengal across different geographical zones since last two decades, a specimen was collected and identified as *Tulostoma squamosum* (J.F. Gmel.) Pers. from Darjeeling Hills. Geographically, Darjeeling Hills falls under the eastern Himalayan range and encompasses an area of 524,190km^2 (21.95–29.45 ^{0}N & 82.70–100.31 ^{0}E). The forest of the region is mostly dominated by plants like *Castanopsis* sp., *Quercus* sp., *Cryptomeria japonica*, *Alnus* sp., *Magnolia campbellii, Lithocarpus* sp., *Abies* sp., and large *Rhododendron* spp. (State Forest Report 2011–2012; Paloi et al. 2015).

Currently, there are 24 reported species of *Tulostoma* from India, viz.: *T. albiceps* Long & S. Ahmad, *T. albocretaceum* Long & S. Ahmad, *T. amnicola* Long & S. Ahmad, *T. balanoides* Long & S. Ahmad, *T. cineraceum* Long, *T. crassipes* Long & S. Ahmad, *T. evanescens* Long & S. Ahmad, *T. exitum* Long & S. Ahmad, *T. hygrophilum* Long & S. Ahmad, *T. inonotum* Long & S. Ahmad, *T. membranaceum* Long & S. Ahmad, *T. mussooriense* Henn., *T. operculatum* Long & S. Ahmad, *T. parvissimum* Long & S. Ahmad, *T. perplexum* Long & S. Ahmad, *T. pluriosteum* Long & S. Ahmad, *T. psilophilum* Long & S. Ahmad, *T. puncticulosum* Long & S. Ahmad, *T. pygmaeum* Lloyd, *T. sedimenticola* Long & S. Ahmad, *T. subsquamosum* Long & S. Ahmad, *T. volvulatum*

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Borshchov var. *volvulatum*, *T. vulgare* Long & S. Ahmad, and *T. wightii* Berk. (Wright 1987). The present study reports *Tulostoma squamosum* for the first time from India based on morphological as well as molecular data along with comparison of morphologically and phylogenetically related species. In addition, the sequence of the Indian collection was compared to the sequences, deposited from other regions of the world, to find out the changes of the nucleotide positions and evolutionary divergence.

Materials And Methods

Morphological protocols

Fresh basidiomata were collected from Darjeeling Hills of West Bengal, India during the month of July 2019. Field photographs of the fresh basidiomata were taken at the field with Canon EOS 1200D (Canon, India) camera. For colour notations, Kornerup & Wanscher (1978) was followed. Collected basidiocarps were dried with a field drier at 50–60 °C.

For microscopic observations, free-hand sections were prepared from the dried basidiomata and 5% KOH solution was used to revive those hand-made sections. After staining with Congo red, and Melzer's reagents, sections were observed with Dewinter 'crown' trinocular microscope (Dewinter Optical Inc., New Delhi). Spores were measured with atleast 20 measurements from each of the collected three basidiocarps. In spore statistics, values in parentheses represent minimum or maximum measured values; X_m denotes the mean of the spore length by its width (± standard deviation); Q represents range variation of the quotient of basidiospore length/width ratio in any one basidiospore; Q_m, the mean of Q-values (± standard deviation); and n, the total number of spores measured. For future reference, voucher specimens were deposited in the Calcutta University Herbarium (CUH).

DNA extraction and PCR amplification

Genomic DNA was extracted from the dried fruitbodies following Dutta et al. (2018). PCR amplification of the nuclear ribosomal internal transcribed spacer sequence (nrITS) region was performed using fungal universal primers pair ITS1 and ITS4 (White et al. 1990) on an Applied Biosystems 2720 automated thermal cycler using the thermal profile as described by Dutta et al. (2018). After purification by QIAquick® Gel Extraction Kit (QIAGEN, Germany), PCR products were subjected to automated DNA sequencing on ABI3730xl DNA Analyzer (Applied Biosystems, USA) using the same primer pairs used for the amplification of rDNA ITS region.

The newly generated sequence of *T. squamosum* was then edited using BioEdit v7.0.5 software (Ibis Therapeutics, Carlsbad, CA) and used for a BLAST search in the NCBI database. Altogether 36 nrDNA ITS sequences of *Tulostoma* representing 28 species were chosen for the phylogenetic analyses based on the BLAST search and the previous study of Jeppson et al. (2017). *Lycoperdon perlatum* Pers. and *Calvatia gigantea* (Batsch) Lloyd were selected as out-group taxa for rooting purpose following Jeppson et al. (2017).

Sequence alignment and phylogenetic analyses

The nrITS data set was aligned using MAFFT v.7.402 (Katoh & Standley 2013) on XSEDE in the CIPRES web portal (http://www.phylo.org/portal2/) (Miller et al. 2010). The aligned datasets were then imported to MEGA v.7.0 (Kumar et al. 2016) for additional manual adjustments.

Statistical selection for the best fit model of nucleotide substitution for the dataset was performed by jModelTest2 (Darriba et al. 2012) on XSEDE using CIPRES web portal. For the given dataset, GTR+G model was selected as the best fit model for the phylogenetic analyses based on the lowest BIC values of 12712.992931.

Maximum likelihood bootstrapping analyses were performed with RAxML-HPC2 v. 8.2.12 (Stamatakis 2006), using the model parameters as suggested by jModelTest2 on the CIPRES NSF XSEDE resource with bootstrap statistics calculated from 1,000 bootstrap replicates.

Bayesian inference (BI) of the phylogeny were carried out using MrBayes v.3.2.2 (Ronquist et al. 2012) using metro-polis-coupled Markov chain monte carlo analyses (Geyer 1991). The general time reversible (GTR) model was employed with gamma-distributed substitution rates. Markov chains were run for 10^6 generations, saving a tree every 100^{th} generation. Default settings in MrBayes were used for the incremental heating scheme for the chains (3 heated and 1 cold chain), branch lengths (unconstrained: exponential (10.0)), partition-specific rate multiplier (fixed (1.0)), and uninformative topology (uniform) priors. After burn in initial 25% trees, MrBayes was used to compute a 50% majority rule consensus of the remaining trees to obtain estimates of the posterior probabilities (PPs) of the groups. Maximum likelihood bootstrap (MLBS) and Bayesian posterior probabilities (PP) values over 50% and 0.50 are reported in the resulting tree.

Nucleotide sequence comparison of *T. squamosum* across different geographic origins

Based on the earlier published studies (Hussain et al. 2016, Jeppson et al. 2017), three well representative sequences of *Tulostoma squamosum*, deposited based on the collections made from different geographic regions, were procured from the NCBI GenBank nucleotide database and were aligned with the newly amplified Indian collection of *T. squamosum* using MUSCLE (Edgar 2004). The nucleotide sequence comparison was accomplished from this alignment for finding out the positional dissimilarities in the entire nrDNA ITS sequence.

Estimates of evolutionary divergence between *Tulostoma squamosum* sequences

Estimation of evolutionary divergence was performed between four sequences of *T. squamosum*, one from the present Indian collection (this study) and the remaining three from France (KU519097), Pakistan (KT285883), and Spain (KU519096). Evolutionary divergence analysis was carried out in MEGA v.7.0 (Kumar et al. 2016) using the Kimura 2-parameter model (Kimura 1980) where all positions containing gaps and missing data were eliminated.

Results

Phylogenetic analyses

Sequencing product of the Indian collection of *Tulostoma squamosum* ranged 658 nucleotides. ITS sequences were aligned and the ends trimmed to create a dataset of 726 base pairs of which the final alignment had 420 distinct alignment patterns. Bayesian analyses reached a standard deviation of split frequencies of 0.002 after 10^6 generations and the credible sets of trees included 7,535 trees after excluding the preliminary 25% trees as the burn-in. The trees generated using the ML and Bayesian analyses were identical in topology. Therefore, only the phylogenetic tree generated using ML analysis (InL = -6084.179608) is shown in Figure 1.

Nucleotide sequence comparison

Comparison made from the alignment of an entire nrDNA ITS region of the Indian sequence of *Tulostoma squamosum* along with the three deposited sequences of the same taxon from France (KU519097), Pakistan (KT285883), and Spain (KU519096) reveals that the Indian collection differs from Pakistani collection by eight nucleotide positions, France and Spain collections by five nucleotide positions each (Table 1).

Addition of two adenine nucleotides were also observed at the 584 and 585 nucleotide positions for the Pakistani sample when compared to the present Indian as well as those of the France and Spain samples. Besides, the Indian collection of *T. squamosum* shows insertion of Thymine nucleotide at the 486 nucleotide position when compared to that of the France, Spain, and Pakistan collections.

Estimation of evolutionary divergence between *Tulostoma squamosum* sequences

Estimation of Evolutionary Divergence of four sequences of *Tulostoma squamosum* from India (this study, MN809136), France (KU519097), Pakistan (KT285883) and Spain (KU519096) involved a total of 301 positions in the final aligned dataset. The present Indian sequence of *T. squamosum* varies by 3.1% from the Pakistani sequence and by 2% from the sequences deposited from France and Spain respectively (Table 2). The Pakistani *T. squamosum* sequence, however, showed variation of 1.7% each from France and Spain *T. squamosum* sequences (Table 2).

Taxonomy

***Tulostoma squamosum* (J.F. Gmel.) Pers.**, Syn. meth. fung. (Göttingen) 1: 139 (1801) (Image 1)

Spore-sac 20–30 mm diam., globose, smaller compared to length of stalk. Exoperidium thin, membranous, greyish-orange (5B3, 5B5-6) towards mouth, elsewhere yellowish-brown (5D5-6; 6E6-8), smooth to obscurely reticulate. Endoperidium

Table 1. Comparison of the entire nrDNA ITS sequences (641 nucleotides) between the Indian collection of *Tulostoma squamosum* (in bold front) and of three sequences of Tulostoma squamosum deposited in GenBank database from France, Pakistan and Spain.

Name of the taxon	Geographic origin	Positions in the ITS 1+2 alignment (641 nucleotides)								
		448	502	503	505	556	610	614	615	635
T. squamosum (MN809136)	India	T	T	A	T	T	C	T	T	A
T. squamosum (KU519097)	France	C	C	A	A	T	C	T	C	G
T. squamosum (KT285883)	Pakistan	C	T	G	A	A	A	C	C	G
T. squamosum (KU519096)	Spain	C	C	A	A	T	C	T	C	G

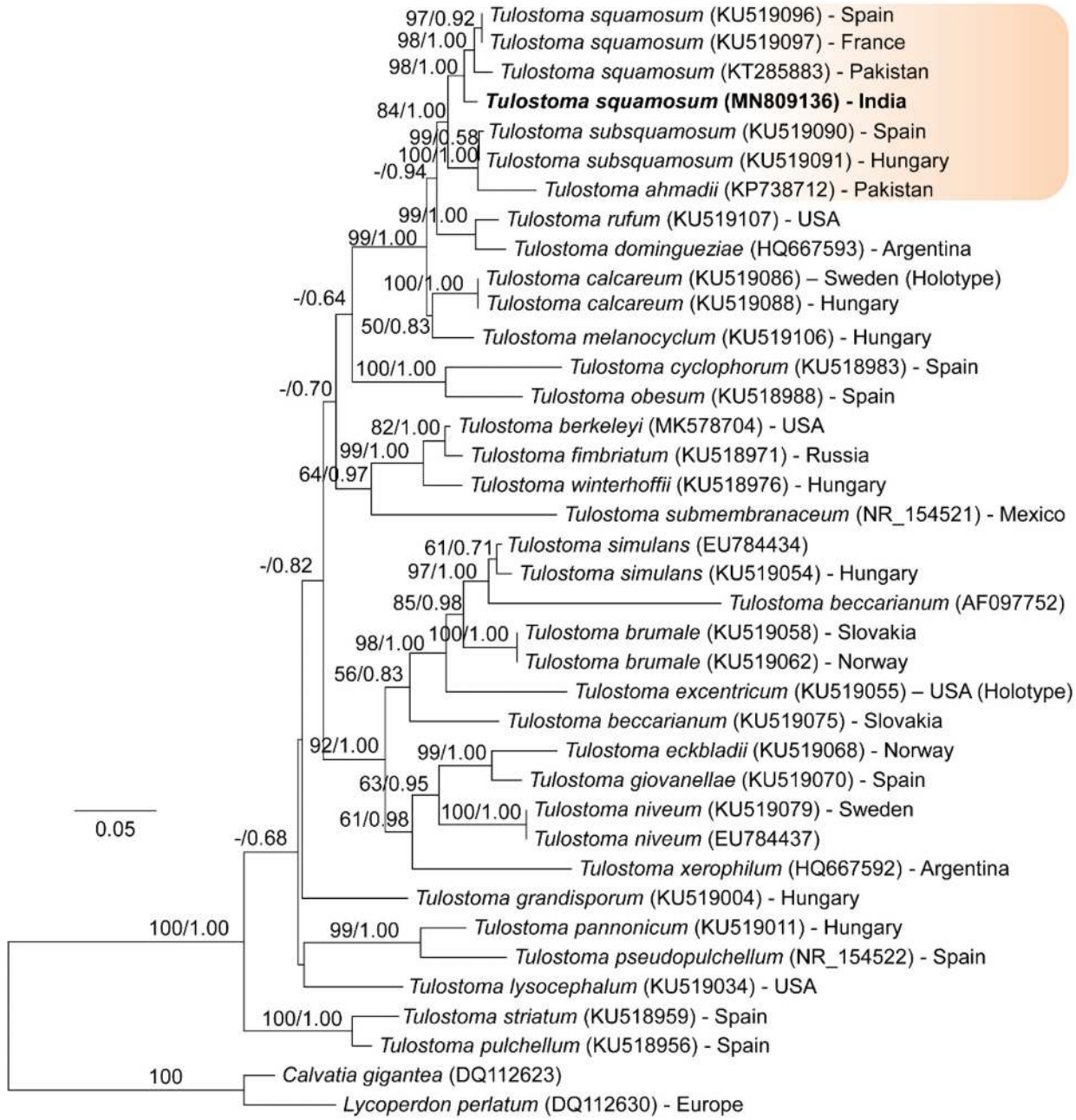

Figure 1. Maximum likelihood tree (InL = -6084.179608) generated using GTR+G model of nucleotide evolution based on newly generated sequence of *Tulostoma squamosum* and acquired nrDNA ITS sequences based on the previous study of Jeppson et al. (2017). *Lycoperdon perlatum* and *Calvatia gigantea* were selected as out-group taxa for rooting purpose following Jeppson et al. (2017). Numbers to the left of / are ML bootstrap percentages (MLBS), and those to the right are Bayesian posterior probabilities (PP). MLBS values ≥ 50% and PP values ≥ 0.50 are shown above or below the nodes. Scale bar represents the expected changes per site.

slightly paler, smooth. Mouth prominent, 1mm diam., somewhat tubular, peristome pale orange (6A3). Socket distinctly separated from stem. Gleba light ochraceous. Stalk 100–120 × 3–6 mm, brown (7D8), sub-scaly to distinctly scaly, scales appressed, mycelial rhizo-morphs present at base.

Spores (6.0–)6.5–7.2(–8.0) × (4.8–)5.2–7.0(–7.2) µm [X_m = 6.82 ± 0.8 × 5.8 ± 0.9 µm, Q = 1.1-1.25, Q_m = 1.18±0.04, n = 60 spores], yellowish-brown, globose to subglobose, oil granules present when viewed with KOH, apiculus short, echinulate ornamentation composed of low (up to 0.4µm) to high (up to 1.2µm) spines, apex obtuse, never reticulate. Basidia not observed. Capillitium hyphae 4.0–8.0 µm broad, interwoven,

Table 2. Genetic divergence matrix among four *Tulostoma squamosum* sequences based on nrDNA ITS sequences data.

GenBank accession no.	Geographic region	MN809136	KU519097	KT285883	KU519096
		India	France	Pakistan	Spain
MN809136	India	-			
KU519097	France	0.020	-		
KT285883	Pakistan	0.031	0.017	-	
KU519096	Spain	0.020	0.000	0.017	-

Image 1. *Tulostoma squamosum* (CUH AM696): A—field photograph of the basidiocarps | B—region of spore-sac attachment to the stem | C—detail of spore-sac showing tubular mouth | D—stalk surface | E—capillitium | F—spores | G—clamped hyphae (Scale: a = 20mm, b–c = 10mm, d = 20mm, e–f = 10µm, g = 20µm). © Arun Kumar Dutta.

hyaline, light yellow to brownish with KOH, septate, branched, thick-walled, lumen visible to lacunar. Gleba composed of more or less loosely arranged, 6.0–12.0 µm broad, interwoven, branched, septate hyphae, lumen distinctly visible, hyphal end clavate to subclavate or sometimes cylindrical, wall 0.4–0.8 µm thick. Stalk surface hyphae 6.0–9.0 µm broad, tightly arranged, hyaline, septate, oil granules present when viewed with KOH, thin-walled.

Habit and habitat: Solitary, scattered, in dead and decomposed leaf litter mixed soil among *Quercus* vegetation.

Known distribution: Europe, North America, Germany (Esqueda et al. 2004), Turkey (Sesli et al. 2000), Pakistan (Hussain et al. 2016), and now India (this study).

Specimen examined: AKD 3/2019 (CUH AM696), 08.vii.2019, India: West Bengal, Darjeeling District, beside Raj Bhavan, 27.051^{0}N & 88.262^{0}E, 2,105m elevation, coll. A.K. Dutta & S. Paloi.

Remarks: *Tulostoma squamosum* is morphologically characterized by the presence of a long, scaly stalk coloured reddish-brown, a spore sac (20–30 mm diam.) with a prominent tubular mouth, spores with echinulate ornamentation, membranous exoperidium and pale yellowish-brown endoperidium. Considering the membranous nature of the exoperidium and presence of tubular mouth, *Tulostoma squamosum* is categorized under the Sect. *Brumalia* Pouzar (Pouzar 1958).

Discussion

Tulostoma squamosum was originally described based on the collection made from Germany and later, Persoon (1801) designated the lectotype of the taxon based on his collection from Italy. The present Indian collection of *T. suqamosum*, however, matches well with that of the original description but, differs in having a larger basidiocarp with spore-sac measuring up to 30mm diam. and stalk 110–120 mm long; and larger spores (6.0–8.0 × 5.2–7.2 µm vs. 5.4–6.5 × 4.7–5.8 um).

The phylogenetic analysis based on nrITS region sequence data placed the present Indian collection along with the sequence of the same taxon collected from Spain, France, and Pakistan with strong statistical support values (98% BS, 1.00 PP; Fig. 1) suggesting all of them to be the morphotype of *Tulostoma squamosum*.

Among morphologically related taxa: *Tulostoma brumale* Pers. has an exoperidium coloured light brownish to cinereous brown outside and whitish inside, shorter stalk measuring 14–45 × 1.5–4 mm, coloured straw yellow to light brown with a peculiar sheen, and smaller spores with a mean of 5µm diam. with surface composed of small disperse verrucae (Wright 1987). *Tulostoma dumeticola* Long differs by having somewhat velvety exoperidium consisting of hyphae forming small tuberculate patches, circular mouth, and presence of anastomosed spines on the spore surface forming almost reticulate appearance (Wright 1987). *Tulostoma dennisii* has globose-depressed spore-sac, scaly exoperidium, small bulbous stalk base, and presence of mycosclereids (Wright 1987). The South American species, *T. bruchi* Speg. differs from *T. suqamosum* by its circular mouth, rugose stalk surface, and large papillate spores (Wright 1987).

Among phylogenetically close taxa (Fig. 1), *T. subsquamosum*, earlier reported to occur in India, has thin-scaly exoperidium, circular mouth, a socket that is separated from the stalk by a lacerated membrane, and presence of longer spines (4.6–6.1 µm diam.) as spore ornamentation (Wright 1987). *Tulostoma ahmadii*, described from Pakistan in the recent past (Hussain et al. 2016), differs by its light olive brown exoperidium, pinkish endoperidium, a socket that is composed of dentate and concentrically arranged membranes, presence of a much smaller stalk (30–40 mm long vs. 100–120 mm long), and somewhat larger spores with an average of 9.36 × 7.99 µm.

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