TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2016) 40: 218-225

doi:10.3906/bot-1501-9

Tulostoma ahmadii sp. nov. and *T. squamosum* from Pakistan

Shah HUSSAIN[1], Nousheen YOUSAF[2,*], Najam-ul-Sehar AFSHAN[3], Abdul Rehman NIAZI[2], Habib AHMAD[4], Abdul Nasir KHALID[2]

[1]Department of Botany, Hazara University, Mansehra, Pakistan
[2]Department of Botany, University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan
[3]Center for Undergraduate Studies, University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan
[4]Department of Genetics, Hazara University, Mansehra, Pakistan

Received: 06.01.2015 • **Accepted/Published Online:** 15.07.2015 • **Final Version:** 09.02.2016

Abstract: *Tulostoma ahmadii*, a new species found in the subtropical pine forest of Malakand, Pakistan, is described and illustrated. It is characterized by parallel to squamulose deep reddish brown scales on stipe with bulbous base, warty exoperidium, and tubular and relatively large-sized basidiospores. Macro- and microscopic features along with sequence data demonstrated that *Tulostoma ahmadii* is a distinct species from other members of *Tulostoma*. *T. squamosum*, which is represented here as a new record for the country, has also been characterized on a molecular basis.

Key words: ITS, Malakand, new species, stalked puffballs, *Tulostoma*

1. Introduction

The genus *Tulostoma* Pers. is a large group of gasteroid fungi, commonly called stalked puffballs, belong to the family Agaricaceae (Agaricales: Basidiomycota). This genus is characterized by a rounded spore case with apical mouth attached to a stipe. It is represented by 81 established species (Kirk et al., 2008; Caffot et al., 2011) but more than 100 names for this group have been cited in the literature (Wright, 1987; Moreno et al., 1992, 1995, 2002; Fan and Liu, 2005). The distribution of these fungi is worldwide, but they prefer xeric, warm, and sandy habitats (Wright, 1987; Esqueda-Valle et al., 2000). Most of the taxa of this genus are found throughout the year.

Taxonomy of the genus *Tulostoma* was previously based on morphological features like size, color, and shape of basidiomata as well as microscopic characters like shape, size, and color of eucapillitia, paracapillitia, and basidiospores (Wright, 1987). During the last decade, taxonomy of these fungi has been analyzed through molecular tools such as sequencing of the nrDNA gene, RPB2 gene, and translation elongation factor 1-alpha-like gene (Bellemain et al., 2010; Caffot et al., 2011).

Previously, 24 taxa in the genus *Tulostoma* have been reported from Pakistan (Ahmad et al., 1997). In the present study, a few basidiomata of *Tulostoma* species have been collected from Malakand of Khyber Pakhtunkhwa and Khushab districts of Punjab, Pakistan. Macromorphology, microscopic analysis, and ITS sequence data strongly support that *Tulostoma ahmadii* is a new species. *T. squamosum* Pers. is described here as a new record for Pakistan.

This work is a continuation of efforts for exploring biodiversity of gasteroid fungi from different regions of Pakistan.

2. Materials and methods

2.1. Collection and processing of samples

Specimens were collected, photographed, vouchered, dried using a fan heater, and kept in paper boxes. For basidiomata color designations, the Munsell (1975) color system was followed. Slides were prepared from exoperidium, endoperidium, and gleba in 5% KOH, lactic acid, and Trypan blue. The exoperidium was observed by scratching the outer surface of the spore case and stipe with a needle under a dissecting microscope (Meiji Techno, Japan). Microscopic features were observed under a binocular microscope (MX4300H, Meiji Techno Co., Ltd., Japan). Basidiospore dimensions were based on the measurement of at least 20 randomly selected spores of 2 basidiomata of each collection by using the formula (n/m/p), where n is number of spores measured from m number of specimens out of p number of collections. Average length and width

* Correspondence: nousheenyousaf@gmail.com

of all spores by avX, quotient length by width of spores by Q, and average quotient by avQ are provided (Liang et al., 2011). Color reactions of spores were studied in Melzer's reagent (Largent et al., 1977) and line drawings were made with a camera lucida.

2.2. DNA extraction, PCR amplification, and DNA sequencing

Genomic DNA was extracted using the DNeasy Plant Mini Kit by QIAGEN (Cat. No. 69104) following standard manufacturer's protocol and by using modified the CTAB method following the Bruns (1995) protocol with small modifications. The ITS-nrDNA region was amplified using the universal primer pair ITS1F and ITS4 (White et al., 1990). Polymerase chain reaction (PCR) was performed in a reaction volume of 20 µL containing 10 µL of 2X PCR buffer (Sigma-Aldrich Co. LLC), 0.1 µL of each primer, 8.8 µL of deionized distilled water, and 1 µL of DNA template. PCR parameters for one cycle are 94 °C for 1 min and 35 cycles at 94 °C, 53 °C, and 72 °C each for 1 min, with a final extension at 72 °C for 8 min. The amplified products were run through 1% agarose gel stained with ethidium bromide and visualized under the UV Light Gel Documentation System (UVtec, Avebury House, Cambridge, UK), using default settings. The PCR products were directly sequenced in both directions using the same primer set with the Automatic Sequencer 3730XL (Macrogen, South Korea).

Sequences were manually edited and assembled using BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html). All sequences generated for this study were submitted to GenBank and the accession numbers for these as well as for other closely related taxa used in the phylogenetic analysis are cited in the Table. Multiple sequence alignment was performed using MUSCLE alignment software (Edgar, 2004). Phylogenetic trees were constructed with the maximum likelihood algorithm based on the Jukes and Cantor (1969) model of sequence evolution using default settings of MEGA6 software (Tamura et al., 2013). A bootstrap consensus tree was inferred from 1000 replicates, and corresponding bootstrap values of >50% are cited in the tree. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log likelihood value.

Table. ITS-rDNA sequences included in phylogenetic analyses for *Tulostoma* and related species.

Taxa	Origin	GenBank Accession no.	Voucher ID
Lycoperdon perlatum	Virginia	AY264919	-
Tulostoma ahmadii	Pakistan	KP738711	SH-33
T. ahmadii	Pakistan	KP738712	SH-33
T. beccarianum	Spain	AF097752	BCC-MPM 1663
T. brumale	England	EU784433	RBG Kew K(M)106304
T. brumale	England	EU784434	RBG Kew K(M)109188
T. domingueziae	Japan	HQ667593	MLHC3 (CORD)
T. domingueziae	Japan	HQ667594	MLHC24 (CORD)
T. kotlabae	Sweden	MJ6623	MJ6623
T. melanocyclum	England	EU784435	RBG Kew K(M)64453
T. melanocyclum	England	EU784436	RBG Kew K(M)75782
T. niveum	England	EU784437	RBG Kew K(M)19293
T. squamosum	Pakistan	KT285883	TPK2
T. squamosum	Sweden	DQ415732	Mrazek1300
Tulostoma sp.	Japan	HQ667595	MLHC200 (CORD)
Tulostoma sp.	Japan	HQ667596	MLHC210 (CORD)
T. xerophilum	Argentina	HQ667592	MLHC212 (CORD)

3. Results

3.1. Taxonomy

3.1.1. *Tulostoma ahmadii* Hussain and Khalid sp. nov. (Figures 1 and 2)

Mycobank #: MB 811201

GenBank Accession #: KP738711, KP738712

Diagnosis: *T. ahmadii* characterized by bunch of upright basidiomata on woody underground somewhat flat mycelial cord; warty exoperidium with tubular mouth; parallel to squamulose, deep reddish brown scales over stipe; and relatively large (7–9 × 6.8 µm) globose and subglobose to ellipsoid basidiospores.

Holotype: PAKISTAN, Khyber Pakhtunkhwa, Qaldara Dargai, gregarious, on sandy soil and dead wood, 510 m a.s.l., 31 August 2013, S. Hussain, SH-33 (HU Herbarium No. SH–33).

Etymology: Named after the late Pakistani mycologist S Ahmad.

Figure 1. *Tulostoma ahmadii*: A, B, E–G) Different views of mature basidiomata. C and D) Tubular mouth. H and I) Attachment of spore sac to stipe through socket. J and K) Spores. L) Joint-like septum in eucapillitial hypha. Scale bars: A, B, F = 1.8 cm, C–E = 0.72 cm, G = 1.35 cm, H and I = 2.6 cm, J–L = 0.4 µm.

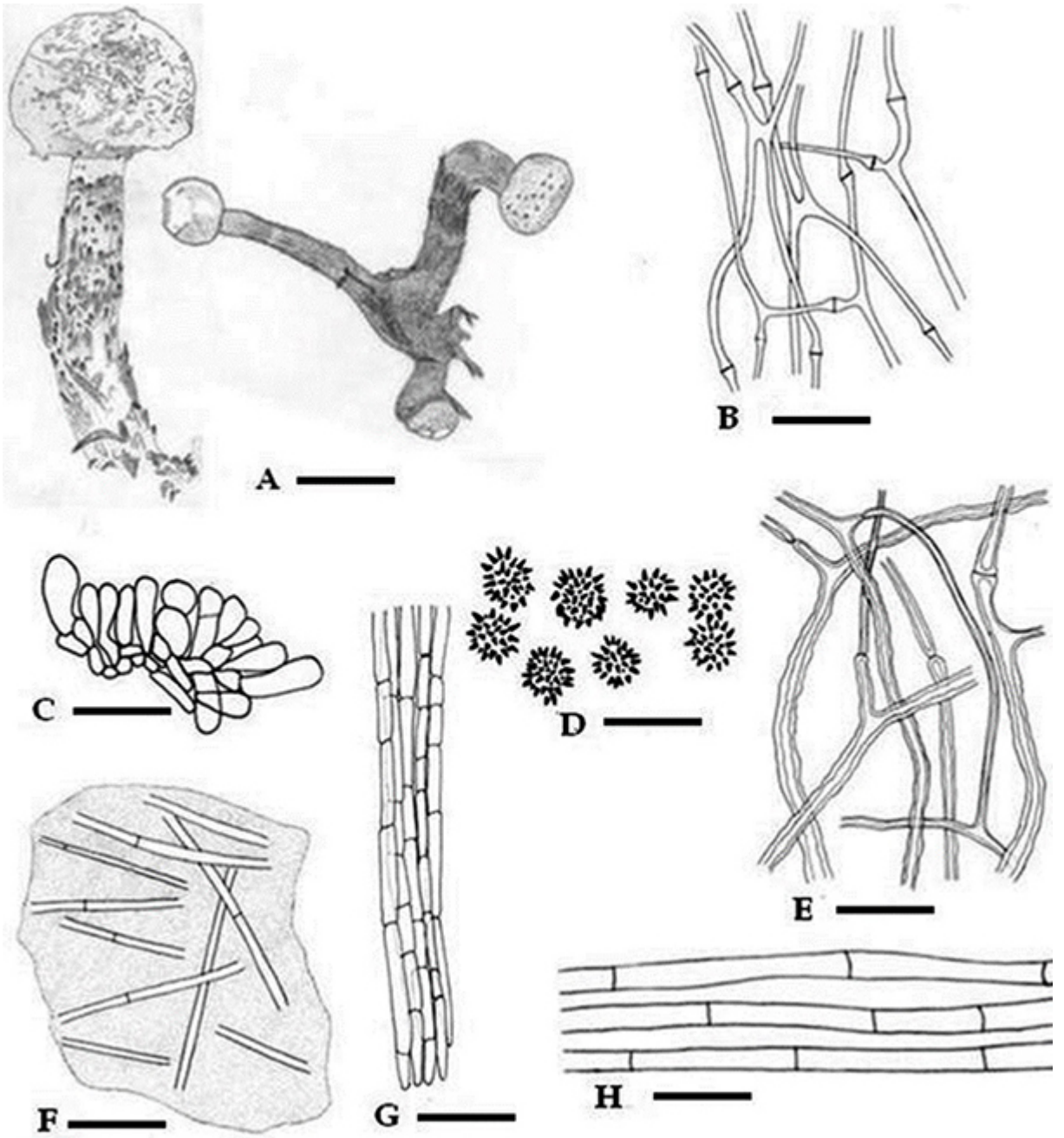

Figure 2. Illustrations of morphoanatomical features of *Tulostoma ahmadii*. A- Basidioma. B- Endoperidial hyphae. C- Exoperidium warts cells. D- Basidiospores. E- Capillitium. F- Exoperidium membrane. G- Socket hyphae. H- Stipe hyphae. Scale bars: A = 2 cm, B = 0.12 µm, C = 0.36 µm, D = 0.25 µm, E = 0.11 µm, F = 0.12 µm, G and H = 0.16 µm.

Sporophore consists of spore case and stipe. ***Spore sac*** 8–15 mm high × 15–20 mm in diam., globose to subglobose. ***Peridium*** double. ***Exoperidium*** membranous to warty, warts light olive brown (2.5Y 5/2–2.5Y 5/2). ***Endoperidium*** papery, pinkish, opens by an apical mouth at maturity; ***Mouth*** circular, tubular, surrounded with white mass of hyphae. ***Socket*** conspicuous, with several dentate concentrically organized membranes of different lengths, the external one hanging from the endoperidium and the longest ones embracing the stem. ***Stipe*** 30–40 × 3–8 mm, cylindrical, slightly tapering towards spore case, hollow, squamous-rugose, scaly; scales striated, thick, strong reddish brown (10R 3/8–10R 3/10) to deep reddish brown (10R 1/10–10R 2/6), sloughs off at several points, from base upwards, base of the stipe bulbous with thin, white rhizoids. Mature fruiting bodies arise in the form

of bunch from the underground woody flat mycelial base. ***Gleba*** orange (5YR 6/12–5YR 7/12).

Basidia rarely observed, 2-spored, ellipsoid to clavate, 23 × 9 µm. ***Eucapillitium*** light green, undulate, rarely branched, septate, thick walled, 6–9 µm in diam., wall thickness 2–3 µm. ***Basidiospores*** [50/4/2], (6–)7.5–9.4(–10.3) × (4–)6.3–8.2(–9.4) µm (avX = 9.36 × 7.99 µm), Q = 1.11–1.36, avQ = 1.17, globose to subglobose, brown in 5% KOH, with small apiculus, ornamented, in the form of thin and wide, solitary and appressed spines.

Exoperidium with two sublayers; *outer layer* composed of subglobose to elongated hyphal cells; 7–10 × 2–4 µm, irregularly arranged; *inner layer* present below the warts, composed of thin, straight, septate hyphae, 3–5 µm. ***Endoperidium*** composed of smooth, long, septate, highly branched hyphae, 3–5 µm, wall thickness 1–2 µm. ***Socket membranes*** composed of cylindrical hyphae, 3–4.5 µm. *Stipe* composed of thin-walled, hyaline, straight and septate hyphae, 4–6 µm. The basal bulb hyphae up to 1–2 µm.

Habitat: Under deciduous trees in humus-rich sandy soil near *Justicia adhatoda* L. along with *Convolvulus* sp.

Specimen examined: PAKISTAN, Khyber Pakhtunkhwa, Qaldara Dargai, 3 km east of Swat-Malakand Highway, 34°52′36.3″S, 71°88′03.5″E, at 510 m a.s.l, sandy soil and dead wood, 31 August 2013 (SH-33), 3 March 2014 (SH-34), S. Hussain and A. N. Khalid, (Holotype HU Herbarium No. SH–33).

3.2. New record

Tulostoma squamosum Pers., Syn. meth. fung. (Göttingen) 1: 139 (1801) (Figures 3 and 4)

Specimen examined: PAKISTAN: Punjab, Khushab district, salt range, Sakesar hills, solitary, on ground under *Pinus rouxburghii,* at 1552 m a.s.l., 10 August 2006, A. N. Khalid, TPK2 (LAH100000167).

T. squamosum has been reported from Turkey, Georgia, India, Sweden, Austria, and Macedonia (Wright, 1987). *T. squamosum* in this study was collected from Sakesar of Khushab district, Punjab, Pakistan. Sakesar is a highest

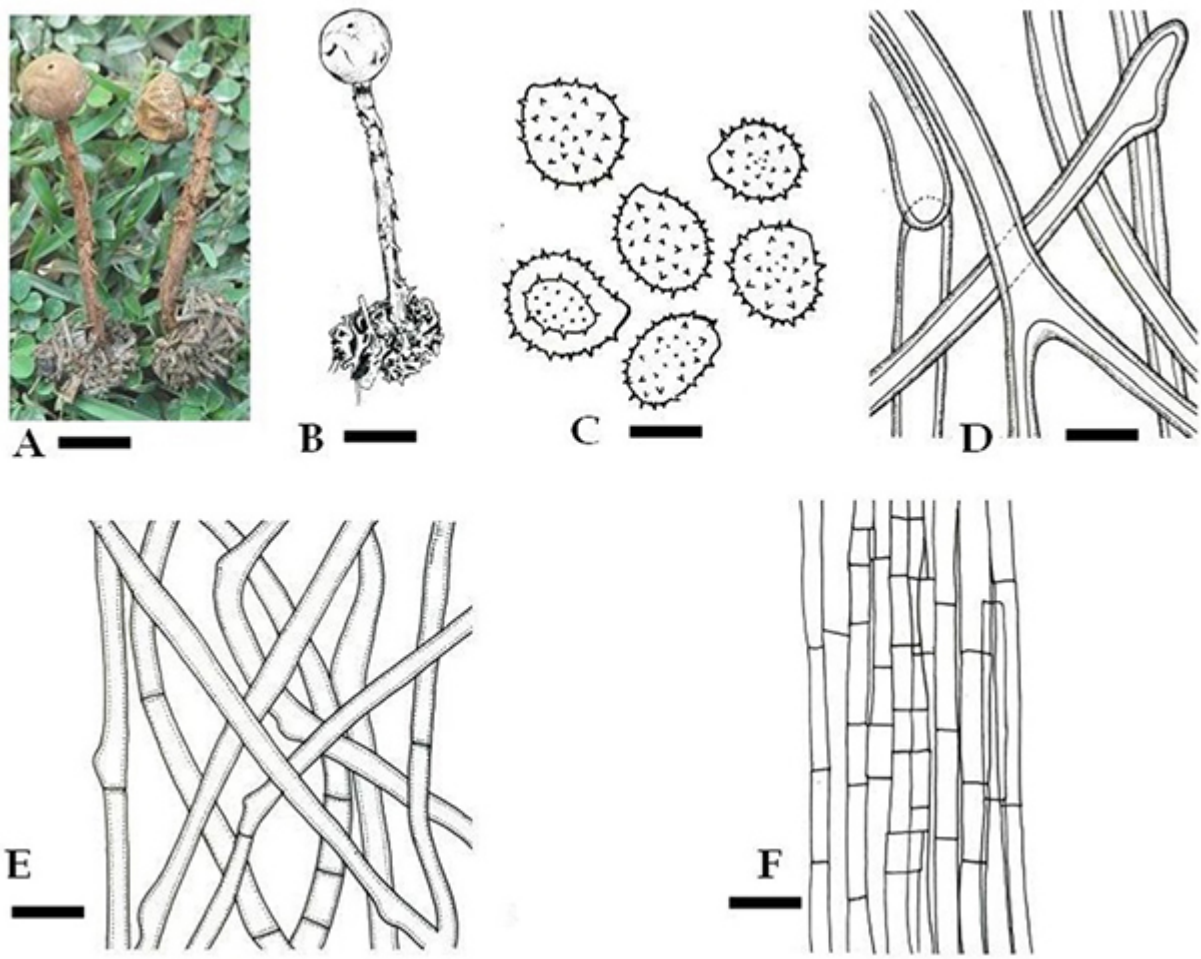

Figure 3. Morphoanatomic features of *Tulostoma squamosum*. A- Mature basidiomata. B- Line drawings of basidioma. C- Basidiospores. D- Eucapillitial hyphae. E- Endoperidial hyphae. F- Stipe hyphae. Scale bars: A and B = 1 cm, C = 4 µm, D–F= 9 µm.

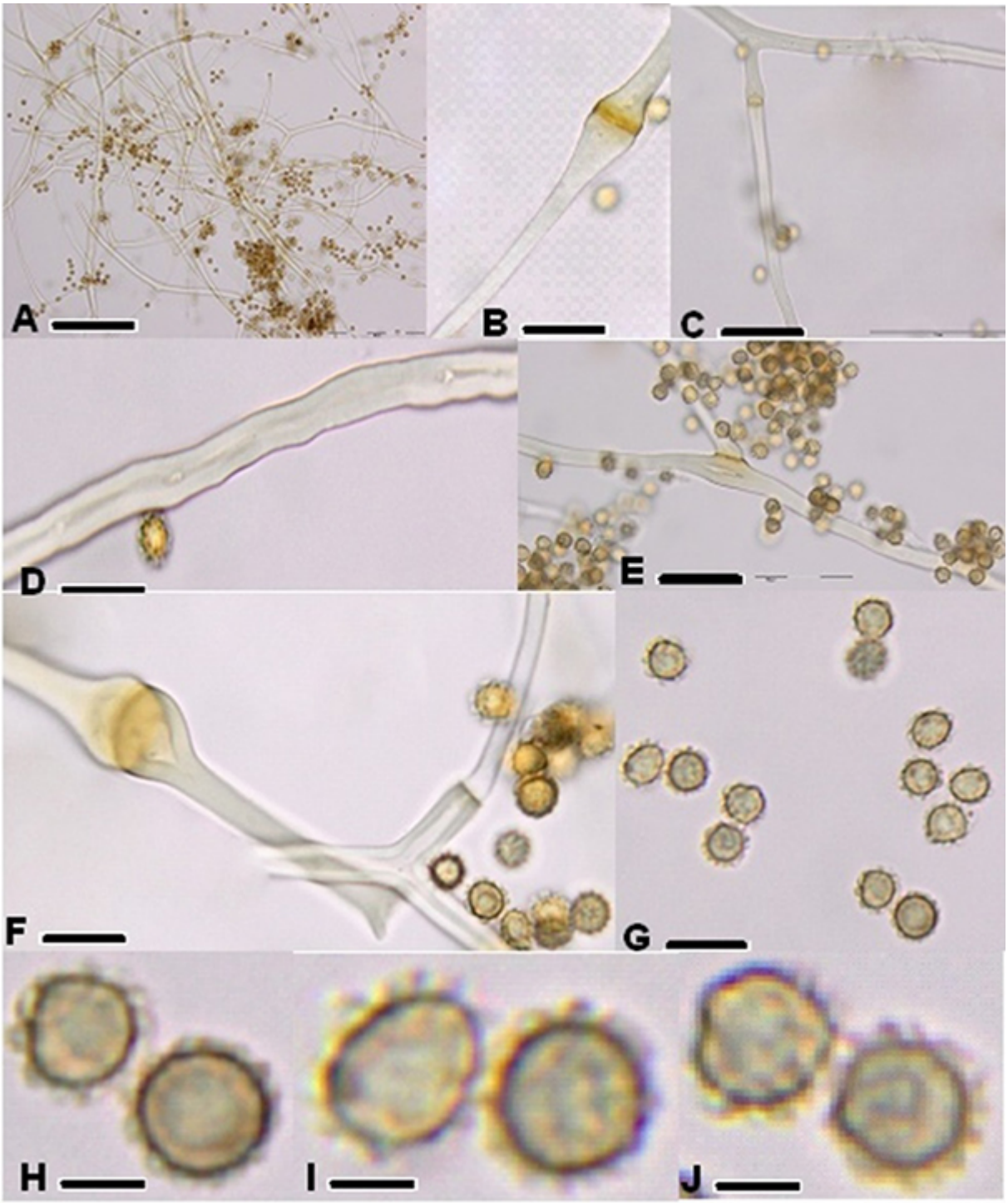

Figure 4. Light microscopy photographs of *Tulostoma squamosum*. A–F- Different views of eucapillitial hyphae. G–J- Basidiospores. Scale bars: A = 10 µm, B = 5.9 µm, C = 3.5 µm, D = 8.2 µm, E = 4.7 µm, F = 11.8 µm, G = 12 µm, H–J = 43.1 µm.

peak of the salt range in Soone valley and its relatively cooler climate is very different from the other habitats in the salt range (Nawaz et al., 2012). It is represented here as a new record for the country.

4. Discussion

Two collections of *T. ahmadii* were made during March and August 2013 and 2014 on sandy soil in a subtropical pine forest of Malakand district, Khyber Pakhtunkhwa.

The Malakand district lies between 34°33′N and 34°56′N and between 71°55′E and 71°52′E, and it features a subtropical climate with heavy rainfall (600–1100 mm annually) (Karim, 2008).

Morphologically it appears close to *Tulostoma domingueziae* Hernández Caffot, *T. dumeticola* Long, *T. exasperatum* Mont., and *T. macalpineanum* Lloyd due to the shared feature of ornamented exoperidium.

Among these, *T. domingueziae* is the closest taxon, recently described from Argentina (Caffot et al., 2011), different from *T. ahmadii* by having reddish brown warty exoperidium that breaks off, cream endoperidium with branched hyphae, 8–9 µm, with yellowish brown, subglobose to irregular spores (5.7–8.7 µm). *T. dumeticola* and *T. exasperatum* has inconspicuous socket with one to no dentate membrane in contrast to *T. ahmadii*, which has a prominent socket with many dentate membranes. It can also be compared with *T. macalpineanum* but it has pinkish white exoperidium and smooth spores versus olive brown exoperidium and warted basidiospores in *T. ahmadii*.

T. squamosum Pers., another related taxon, has smaller basidiospores compared to larger basidiospores of *T. ahmadii* (7.3–8.7 × 6.8–7.8 µm vs 7.5–9.4 × 6.3–8.2 µm).

For phylogenetic analysis, three sequences of the ITS-nrDNA region, two of *Tulostoma ahmadii* (SH-33), comprising 613 bp, and one of *T. squamosum* (762 bp), were generated for this study. Sequences for multiple alignment were obtained from the literature and GenBank (Caffot et al., 2011). The final dataset contains 17 sequences, 16 in-group and 1 out-group sequence. All in-group sequences belong to the genus *Tulostoma* and *Lycoperdon perlatum* (AY264919) was taken as an out-group. There were a total of 810 positions in the final dataset including 150 parsimony-informative and 499 conserved sites. A phylogenetic tree with the highest log likelihood (–3681.5903) is shown. Phylogenetic analysis based on Jukes and the Cantor model of maximum likelihood method shows a distinct position of *T. ahmadii* with moderate bootstrap support.

In the phylogenetic analysis, two clades were resolved based on the shape and ornamentation of the basidiospores. *T. ahmadii* has globose to subglobose spores with verruculose ornamentation and was clustered along with two sequences each of *T. squamosum*, *T. melanocyclum*, *T. domingueziae*, and *Tulostoma* sp. Other taxa in the genus having spores with reticulate ornamentation were grouped in clade II (Figure 5).

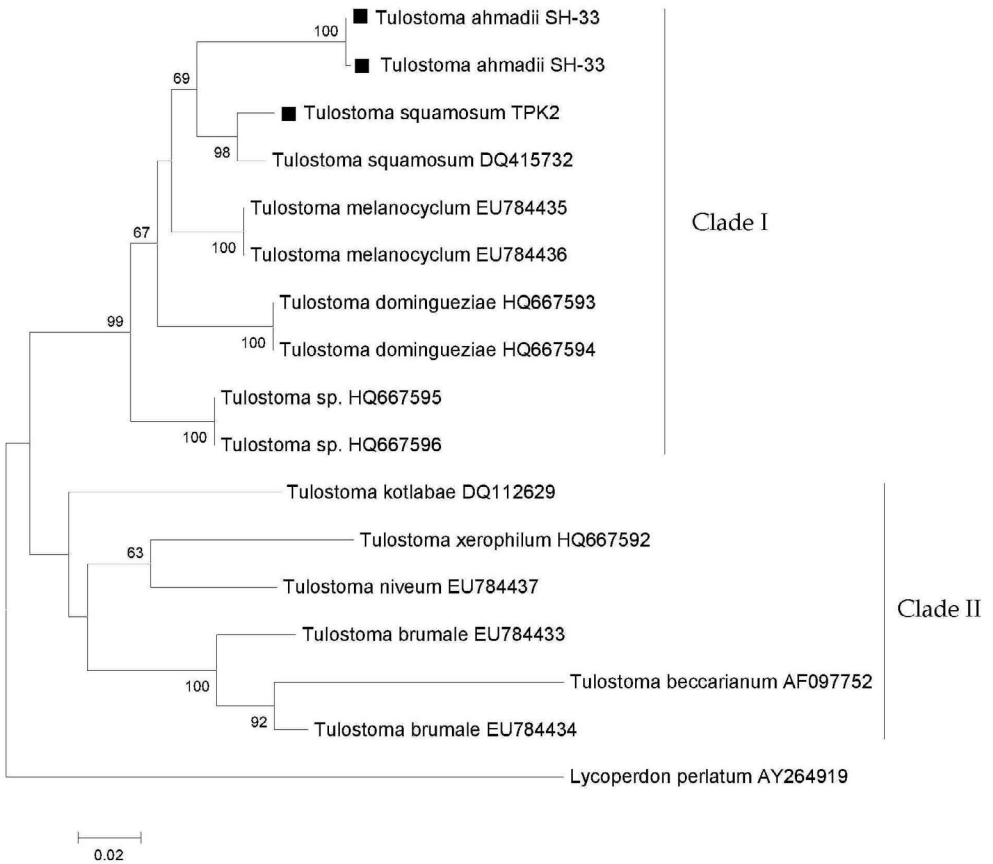

Figure 5. Molecular phylogenetic analysis of *Tulostoma ahmadii* and related taxa by maximum likelihood method. (▪) are sequences generated during this study.

Phylogenetically the closest member of *T. ahmadii* is *T. squamosum*, forming a sister clade with a strongly supported bootstrap value 69%. The latter is different from *T. ahmadii* by having smaller peridium (10 mm) with appressed to imbricate scales to almost smooth stipe, 15 × 2 mm, smaller basidiospores (4–6 µm), dark dirty reddish brown endoperidium, capillitia much branched, up to 15 µm in diameter with wall thickness of 4 µm (Sesli et al., 2000). Phylogenetically both species fall into clade I due to membranous to verrucose exoperidium, although fibrillose to squamulose deep reddish brown scales are the distinguishing character of *T. ahmadii*. Another close relative is *T. melanocyclum*, having hyphal exoperidium with echinulate spores and prominent apiculus as different features, reported from Europe, Asia, North America, and Brazil (Wright et al., 1972; Wright, 1987; Calonge and Wright, 1989).

Phylogeny based on ITS sequences produced 810 sites with maximum likelihood log value (–3681.5903) in the final dataset. In maximum likelihood analysis *T. ahmadii* separated with 100% bootstrap values from all the available sequences of the genus. Similarly, this is consistent with our morphological observations, which obviously demonstrated that *T. ahmadii* is a new species in the genus *Tulostoma*.

Similarly, *T. squamosum* is reported from Pakistan for the first time, characterized morphoanatomically and phylogenetically as an addition to the gasteroid fungi of Pakistan. *Tulostoma* is the largest genus (23 established species and 1 variety) of the gasteroid fungi of Pakistan (Ahmad et al., 1997), presumably due to the climate of the country, which seems best for these taxa. In light of these findings we hypothesized that this region is rich in diversity of gasteroid fungi and specifically the genus *Tulostoma*. This report provides a baseline for the evaluation of biodiversity of *Tulostoma* and the related gasteroid fungi of Pakistan.

References

Ahmad S, Iqbal SH, Khalid AN (1997). Fungi of Pakistan. Lahore, Pakistan: Sultan Ahmad Mycological Society of Pakistan.

Bellemain E, Carlsen T, Brochmann C, Coissac E, Taberlet P, Kauserud H (2010). ITS as an environmental DNA barcode for fungi: An in silico approach reveals potential PCR biases. BMC Microbiol 10: 189.

Bruns TD (1995). Thoughts on the processes that maintain local species diversity of ectomycorrhizal fungi. Plant Soil 170: 63–73.

Caffot MLH, Dominguez LS, Hosaka K, Crespo EM (2011). *Tulostoma dominguezziae* sp. nov. from *Polylepis australis* woodlands in Cordoba Mountains, central Argentina. Mycologia 103: 1047–1054.

Calonge FD, Wright JE (1989). El género Tulostoma Pers.: Pers. (Gasteromycetes) en España. Bol Soc Micol Madrid 13: 119–137 (in Spanish).

Edgar RC (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32: 1792–1797.

Esqueda-Valle M, Perez-Silva E, Herrera T, Coronado-Andrade M, Estrada-Torres A (2000). Composición de gasteromicetos en un gradiente de vegetación de Sonora, México. Anales del Instituto de Biología Serie Botánica 71: 39–62 (in Spanish).

Fan L, Liu B (2005). Two new species of *Tulostoma* from China. Mycosystema 24: 159–61.

Jukes TH, Cantor CR (1969). Evolution of protein molecules. In: Munro HN, editor. Mammalian Protein Metabolism. New York, NY, USA: Academic Press, pp. 21–132.

Karim A (2008). Constituency NA 35, Malakand. Nazar 1: 9–14.

Kirk PM, Cannon PF, Minter DW, Stalpers JA (2008). Aynsworth and Bisby's Dictionary of Fungi. 10th ed. Wallingford, UK: CAB International.

Largent D, Johnson D, Watling R (1977). How to Identify Mushrooms to Genus III: Microscopic Features. Arcata, CA, USA: Mad River Press.

Liang JF, Yang ZL, Xu DP (2011). A new species of *Lepiota* from China. Mycologia 103: 820–830.

Moreno G, Altés A, Ochoa C, Wright JE (1995). Contribution to the study of the *Tulostomataceae* in Baja. California, Mexico. Mycologia 87: 96–120.

Moreno G, Altés A, Wright JE (1992). *Tulostoma pseudopulchellum* sp. nov: (Tulostomatales, Gasteromycetes) and allied species. Mycotaxon 43: 479–86.

Moreno G, Horak E, Altés A (2002). *Tulostoma kreiselii*, a new species from Tunisia, northern Africa. Feddes Repertorium Specierum Novarum Regni Vegetabilis 113: 7–10.

Munsell (1975). Soil Color Charts. Baltimore, MD, USA: Munsell Color Company.

Nawaz T, Hameed M, Ashraf M, Ahmad F, Ahmad MSA, Hussain M, Ahmad I, Younis A, Ahmad KS (2012). Diversity and conservation status of economically important flora of the salt range, Pakistan. Pak J Bot 44: 203–212.

Sesli E, Wright JE, Türkekul İ (2000). The Genus *Tulostoma* Pers.: Pers. (Gasteromycetes) in Turkey. Turk J Bot 24: 269–272.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30: 2725–2729.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR Protocols. San Diego, CA, USA: Academic Press, pp. 315–322.

Wright JE (1987). The genus *Tulostoma* (Gasteromycetes): a world monograph. Berlin, Germany: J. Cramer, pp. 1–338.

Wright JE, Herrera T, Guzman G (1972). Estudios sobre el género *Tulostoma* en México (Fungi, Gasteromyc.). Cienc Mex 27: 109–122 (in Spanish).