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Tulostoma dominguezziae sp. nov. from *Polylepis australis* woodlands in Córdoba Mountains, central Argentina

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Abstract: The new species, *Tulostoma dominguezziae*, is described and illustrated. It was found in *Polylepis australis* woodlands in central Argentinean highlands. *Tulostoma dominguezziae* is characterized by the combination of a warty exoperidium, contorted stipe covered by thin scales that break off, mouth slightly projected and the socket with up to six dentate hanging membranes. DNA sequence data demonstrated that *T. dominguezziae* is distinct from *Tulostoma* species for which sequence data are available.

Key words: altitudinal woodlands, diversity, ITS, nuc-LSU-rDNA, puffball fungi

INTRODUCTION

The increasing interest in fungal biodiversity has heightened the need for more studies on fungal species. Of particular interest and complexity is genus *Tulostoma* Pers. It is widely accepted that *Tulostoma* diversity is higher in arid and semiarid regions (Miller and Miller 1988, Esqueda-Valle et al. 2000). In Argentina this genus is represented by 50 species reported from 21 provinces. Twelve species have been cited from Córdoba Province (Wright 1987a, b; Domínguez de Toledo 1989; Moreno et al. 1992; Altés and Moreno 1993; Nouhra and Domínguez de Toledo 1993; Altés et al. 1996; Daga et al. 2001; Dios et al. 2004).

There are few records of this genus for *Polylepis* wet-high altitude woodlands from Córdoba Province (Hernández Caffot et al. 2008). *Polylepis* Ruiz & Pav. is a genus of approximately 28 tree species in the Rosaceae (Kessler 2006). *Polylepis australis* Bitter grows in NW and central Argentina at 1300–2600 m. *Polylepis* woodlands have been designated endangered ecosystems due to extreme environmental conditions and human activity (Walter and Gillett 1998, UNEP-WCMC 2004, Kessler 2006, Renison et al. 2009). Its distribution is restricted to deep canyons and ravines along water courses where it forms isolated patches (Simpson 1979, Cingolani et al. 2004, Renison et al. 2006).

The present study was undertaken to evaluate the diversity of ''gasteromycetes'' s.l. of *P. australis* woodlands. Some *Tulostoma* specimens were collected; they turned out to be different from the ones already known. Thus, based on morphological macro- and microscopic features and DNA sequence data, *Tulostoma dominguezziae* is described as a new species.

MATERIALS AND METHODS

Study area.—Specimens were collected in *P. australis* woodlands in the Sierras Grandes in Córdoba Province, central Argentina

Processing and examination of specimens.—Holotype and other specimens were dried, kept frozen a week and deposited in the Herbario del Museo Botánico de la Universidad Nacional de Córdoba (CORD). Specimens were studied under stereoscope (Wild M3Z) and optical microscope (Zeiss Axioplan). Sections were mounted in 3% KOH and lactophenol cotton blue for microscopic examinations. Spore dimensions are based on the measurement of at least 15 randomly selected, plus largest and smallest spores from each basidiocarp of each collection. Crystal composition from the plug of the pore was determined with sulfuric acid. Scanning electron microscopy (SEM) of spores was made with a Zeiss LEO 1450VP. Spores were mounted on aluminium stubs, covered with gold with a standard sputter coater. Identification key was made based on bibliography (Wright 1987a, b; Domínguez de Toledo 1989; Moreno et al. 1992; Altés and Moreno 1993; Nouhra and Domínguez de Toledo 1993; Altés et al. 1996; Daga et al. 2001; Dios et al. 2004).

Molecular analyses.—DNA was extracted from gleba tissue of dried fruiting bodies. The powdery spore mass (approximately 30 mg) first was ground with bead beating, following the protocol of Hosaka and Castellano (2008) and Hosaka (2009). Then 1000 mL 2× CTAB buffer with 0.1 M Na_2SO_3 was added directly to the sample tubes. The DNA extraction followed the protocols of Hosaka and Castellano (2008).

DNA sequence data were obtained from the internal transcribed spacer regions (ITS) and large subunit of the

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nuclear ribosomal DNA (nuc-LSU-rDNA). The primer combination of ITS5 and ITS4 (White et al. 1990) was used to amplify the ITS region. To amplify nuc-LSU-rDNA the combination of LR0R and LR5 (Vilgalys and Hester 1990) was used. These primers also were used for cycle sequencing, except that ITS1 (White et al. 1990) was used instead of ITS5. PCR reactions were carried out with 20 μL reaction volumes, each containing 1 μL genomic DNA, 1 μL dNTPs (4 mM each), 1 μl each primer (8 μM), 0.5 units Taq polymerase (TaKaRa, Tokyo, Japan), 2 μL $MgCl_2$ (25 mM), 2 μL bovine serum albumin (BSA). Parameters were one cycle at 94 C for 3 min, 30 cycles at 94 C for 1 min, 51 C for 30 s and 72 C for 1 min with a final extension at 72 C for 15 min. PCR products were electrophoresed in 1% agarose gels stained with ethidium bromide and viewed under UV light. When amplification bands were confirmed PCR products were purified with the ExoSap-IT (Millipore, Molsheim, France) and directly sequenced with the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Norwalk, Connecticut), following the manufacturer's instructions. DNA sequences obtained from this study, as well as data retrieved from GenBank, were combined to construct larger datasets. A total of 14 and 10 sequences, including the outgroup taxon (*Lycoperdon perlatum*), respectively were used to construct the ITS and LSU databases. The choice of outgroup was based on phylogenetic analyses of Matheny et al. (2006) and Larsson and Jepsson (2008). DNA sequences initially were aligned with MUSCLE 3.6 (Edgar 2004a, b), followed by manual alignment in the data editor of BioEdit 7.0.1 (Hall 1999). Ambiguously aligned regions were excluded from analyses. The datasets were analyzed by maximum parsimony (MP). MP analyses were conducted under the equally weighted parsimony criterion, TBR branch swapping, with MULTREES option on, and 1000 replicates of random addition sequence with PAUP* 4.0b10 (Swofford 2002). Support for the individual nodes was tested with bootstrap (BS) analyses under the equally weighted parsimony criterion. BS analyses were based on 1000 BS replicates using the heuristic search option (TBR and MULTREES options on), with 10 random addition sequences.

TAXONOMY

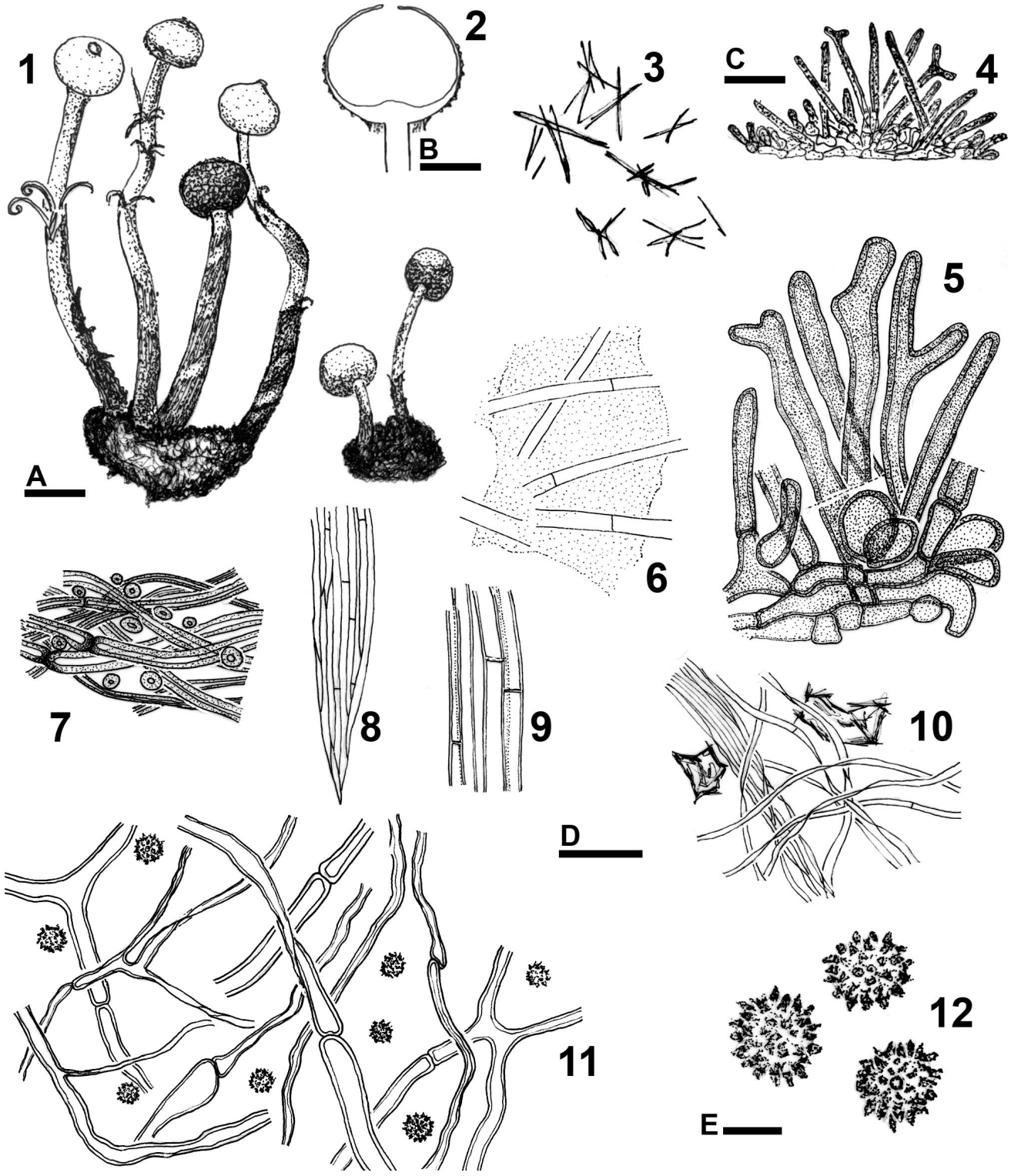
Tulostoma dominguezziae Hernández Caffot sp. nov. FIGS. 1–20

MycoBank MB519370

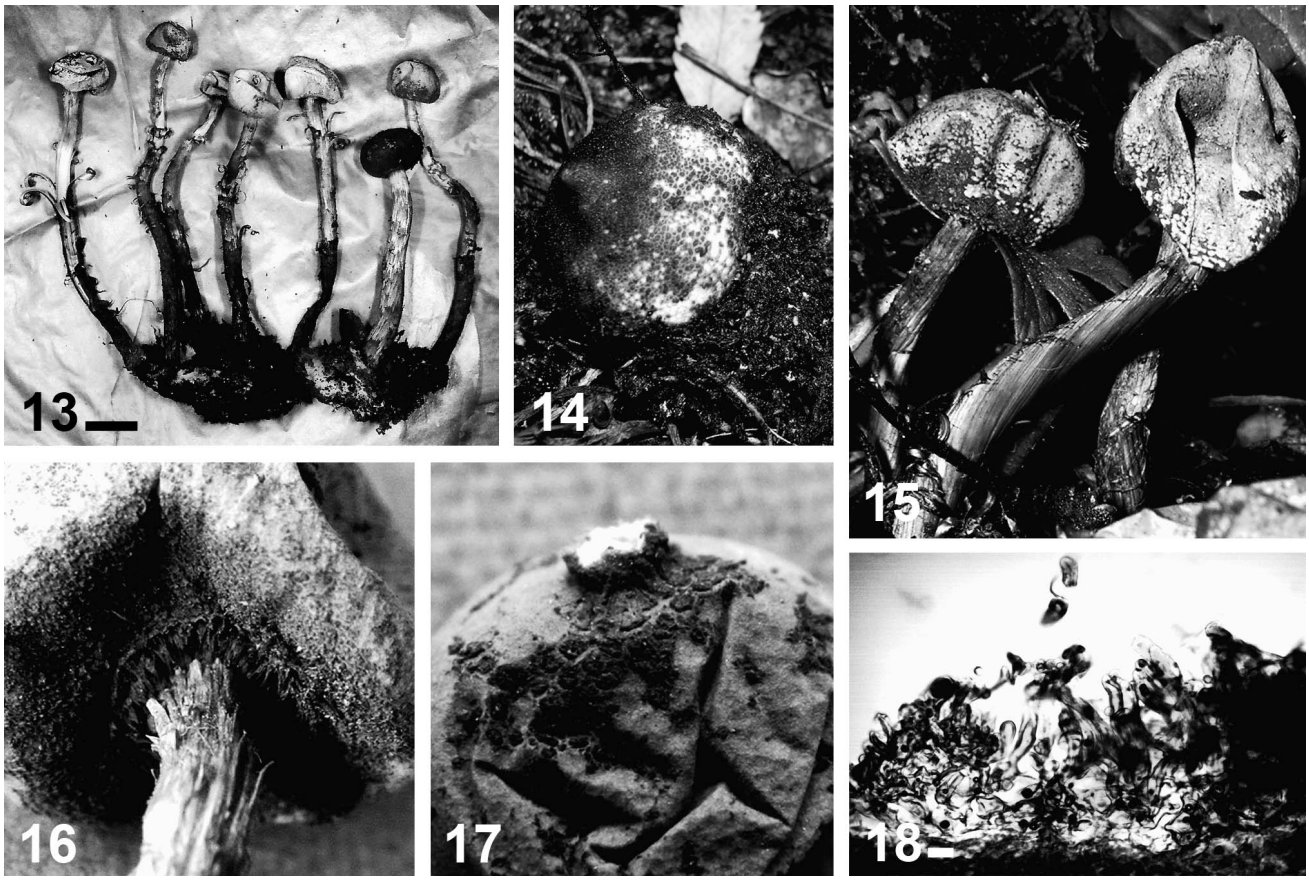
Basidioma usque ad 34–60 mm. Peridio globoso-depresso usque ad 8–9 mm. Exoperidio verrucoso, verrucis ferrugineo-brunneis. Endoperidio membranaceo, glabro, cremeo. Ostiolo rotundato leviter projecto. Collo conspicuo, separato, lacerato ferrugineo-bruneis. Stipite usque 56 × 3 mm, striatulato, ferrugineo, ad basim bulboso. Gleba ochraceofulva. Capillitio usque 8 μm leviter brunneis, ramificato, septato, incrassato ad septos, cum lumen aperto. Sporis globosis vel irregulariter, luteo brunneis, sub microscopio optico (5–)5.7 × 6.25(–8.7) μm, episporio crasso, haud apiculo.

Basidioma 34–60 mm long (FIGS. 1, 13, 14). Sporesac globose to subglobose, up to 4 mm high × 8–9 mm diam (FIGS. 2, 15). Mouth circular, slightly projected, often surrounded by a darker halo, plugged with hyaline, clamped hyphae (up to 6.5 μm diam) intermingled with a white mass of calcium oxalate crystals (FIGS. 3, 17). After the pore opens it is plugged by this white layer, and as the spore sac matures this plug is lost. Exoperidium made up of reddish brownish warts (FIG. 4) at first united by their bases, later fissured basally. Warts discernible with the naked eye, up to 250 μm high and 200 μm diam, on a thin membrane. The base of the warts is formed by hyphae and isodiametric, brownish, thick-walled cells (up to 7.6 μm diam) disorderly arranged; toward the apex are thick walled, light to dark brownish cells (up to 250 high × 11 μm diam), most of them straight and some ramified at the top (FIG. 5). This cell distribution determines the wart pyramidal form (FIG. 18). The thin membrane (8–10 μm formed by collapsed cells) wears off in flakes, leaving a reticulum. The reticulum in mature specimens disappears at the upper part of the spore sac. In some specimens there is a membrane (FIG. 6) over the exoperidium, up to 15 μm thick, formed by a hyaline, straight, septate and thin-walled hyphae of 3–6 μm diam. Endoperidium membranous, smooth, cream-colored formed by septate, long, thick walled hyphae up to 8–9 μm diam (FIG. 7). Socket conspicuous, with up to six dentate concentrically organized membranes of different lengths; the external one hanging from the endoperidium and the longest ones are embracing the stem (FIGS. 8, 16). Stipe in mature specimens contorted (FIG. 15), circular in section, up to 56 mm long × 3 mm diam, wider at the base, ferruginous; its surface breaks off from the apex toward the base, following the grooves, forming thin and long incurved scales, leaving the internal layer exposed, lighter, formed by hyaline, thin-walled and septate hyphae up to 8 μm diam (FIG. 9). In immature specimens it is straight, almost scaly, with the grooves slightly marked. The stipe base usually presents a thick tuft of light brown to hyaline, thin-walled and septate hyphae up to 1–4 μm diam intermingled with debris (FIG. 10).

Gleba pale brownish. Basidia not observed. Capillitium branched, light brown, up to 8 μm diam (FIG. 11), with straight walls regularly thickened, with particles adhered, regular lumen, generally somewhat widened at the septa, which are light to dark brown. Spores subglobose to irregular (FIGS. 12, 19), yellowish-brown under light microscope, up to (5–)5.7 × 6.25(–8.7) μm including the ornamentation, spore wall up to 1 μm, spines up to 1 μm high; apiculus inconspicuous. Under SEM the ornamentation is formed by thin solitary spines and appressed spines forming crests arranged toward the apiculus (FIG. 19).



FIGS. 1–12. *Tulostoma dominguezieae* (MLHC 24, HOLOTYPE). 1. Basidiocarp. 2. Spore sac and socket morphology. 3. Calcium oxalate crystals. 4. Exoperidium wart. 5. Detail of warts cels. 6. Upper exoperidium membrane. 7. Endoperidium detail. 8. Detail of socket dentate membrane. 9. Stipe morphology. 10. Mycelium with debris. 11. Spores and capillitium. 12. Spores. Bars: A = 10 μm, B = 0.5 cm, C = 1 cm, D = 10 μm (valid for FIGS. 3, 7, 8, 9, 10 and 11), E = 5 μm.



FIGS. 13–18. *Tulostoma dominguezeae* (MLHC 24, HOLOTYPE): 13. Basidiocarp. 14. Immature basidioma. 15. Detail of stipe. 16. Detail of the socket. 17. Detail of spore sac and pore. 18. Exoperidium. Bars: 13 = 1 cm, 18 = 10 μm.

HOLOTYPE: ARGENTINA. CÓRDOBA: Dpto. San Javier, Quebrada Los Molles, 31-III-2003, *L.S. Domínguez*, MLHC 24 (CORD).

Etymology.—The epithet *dominguezeae* is the Latin form of Domínguez. This new species is dedicated to our mentor and colleague Dr LS Domínguez.

Habit, habitat and distribution.—Gregarious, fruiting on litter soil. Endemic so far to *P. australis* mature woodlands from Córdoba High Mountains in central Argentina.

Specimens examined: ARGENTINA. CÓRDOBA: Dpto. San Javier, Los Molles, 31-III-2003, *LS Domínguez*, *MLHC 24* (HOLOTYPE, CORD); Quebrada El Tigre, 17-VIII-2003, *GL Robledo*, *MLHC 3* (CORD).

Additional material examined.—Tulostoma dumeticola: ARGENTINA. CÓRDOBA: Dpto. Punilla, Cuesta Blanca, II-1971, *LS Domínguez*, *LSD 1467* (CORD); Dpto. Colón, 16-III-1991, *LS Domínguez*, *LSD 993* (CORD); *Tulostoma sp.*, Dpto. San Javier, Los Molles, (64°56′40″N, 31°59′17″S, 1922 m), 2-II-2007, *ML Hernández Caffot*, *MLHC 200*, *210* (CORD).

COMMENTS

Tulostoma dominguezeae is well characterized by the combination of a reddish brown, warty exoperidium that breaks off, cream endoperidium, subglobose to irregular spores up to (5–)5.7 × 6.25(–8.7) μm, which under SEM have thin verrucose spines that are solitary and form crests toward the apiculus, a contorted and striate stipe, a deep socket with up to six dentate membranes and a mouth plugged by clamped, hyaline hyphae incrusted with fusiforms calcium oxalate crystals.

Within genus *Tulostoma* few species have a warty or verrucose exoperidium. Three species have been cited for Argentina with this type of exoperidium but differ from the new species in these ways: *T. dumeticola* Long is characterized by an exoperidium with smaller warts and a velvety appearance, dark brown endoperidium and an inconspicuous socket with only one dentate membrane and has been cited also for North, Central and South America, Africa and Asia, and found growing on forest soil among litter

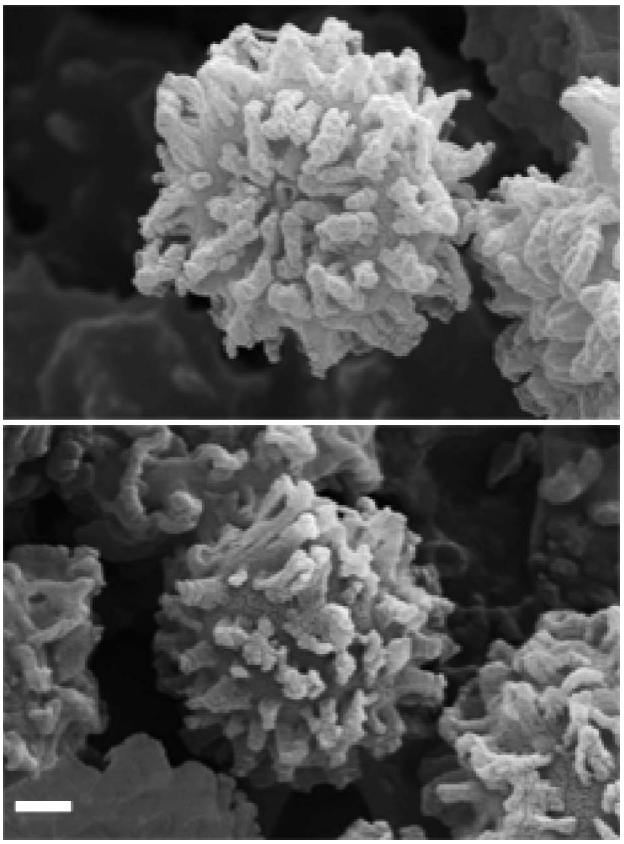


FIG. 19. Spore. Bar: 1 μm.

and humus (Long 1947, Wright 1987a, Guzmán et al. 1992, Daga et al. 2001, Calonge et al. 2007); *T. macalpineanum* Lloyd has a pinkish whitish endoperidium, a socket with one lacerate membrane and almost smooth spores, and it has been cited for Australia and fructifies in sandy soil (Wright 1987a); *T. exasperatum* Mont. has an exoperidium with big spines surrounded by smaller verrucae, a fibrilose-fimbriate mouth, inconspicuous socket without a membrane, a stipe with imbricate scales and reticulated spores, a pantropical distribution and has been found growing on damp wood in tropical rainforests (Long 1947, Wright 1987a, Baseia and Galvão 2002).

On the other hand, *Tulostoma leprosum* (Kalchbr.) Sacc. reported from Australasia is easily distinguished from *T. dominguezziae* because the former has a short and rough stipe and a non-discernible socket (Wright 1987a); *Tulostoma matae* Calonge & J. Carranza from Costa Rica grows on rich soil and has ellipsoidal spores with well developed spines, a visible apiculus and imbricate scales arranged in rings around the stipe (Calonge and Carranza 2003), and *T. dennisii* J.E. Wright from Venezuela and Peru fructifies on naked soil at high altitude (more than 3600 m), has a socket with entire membrane, a stipe with imbricate scales and spores that are echinulate under SEM with some anastomose-forming short crests (Wright 1987a). *Tulostoma pusillum* Berk. differs from *T. dominguezziae* in its distinctive spore ornamentation (pyramidal spines composed of 6–9 columns) and grows in tropical rainforests of North and South America, Asia and Cuba (Wright 1987a).

Other closely related species are *T. melanocyclum* Bres. from Europe, Asia, North America and Brazil that fructify on clayish sandy soils differing from *T. dominguezziae* because of its hyphal exoperidium and echinulate spores with a conspicuous apiculus (Wright et al. 1972, Wright 1987a, Calonge and Wright 1989); *T. squamosum* (J.F. Gmel.) Pers. from Asia, Europe, Africa and North America was found fructifying on calcareous soil among herbaceous vegetation and has a membranous exoperidium that breaks off in small scales, a stipe with appressed and imbricate scales and smaller spores with echinulate and subreticulated ornamentation (Moreno et al. 1984, Wright 1987a, Calonge and Wright 1989, Calonge and Martin 1992, Calonge 1998, Kreisel 2006). *Tulostoma subsquamosum* Long and S. Ahmad differs in its scaly exoperidium, the dirty yellowish white endoperidium and reticulated spores with a conspicuous apiculus, cited from Argentina, Asia, Spain, India, Pakistan and North America, and has been found growing in arid regions with sandy-clayish soil (Wright 1987a, Nouhra and Domínguez de Toledo 1993, Altés et al. 1996, Calonge 1998, Ochoa et al. 1998).

RESULTS AND DISCUSSION

Molecular analyses.—ITS sequences were successfully obtained from *Tulostoma dominguezziae* MLHC 24 (Holotype) and MLHC 3 with GenBank accession numbers HQ667594 and HQ667593 respectively. Additional ITS sequences were obtained from *Tulostoma* sp. (MLHC 200, MLHC 210) and *T. xerophilum* (MLHC 212) with GenBank accession numbers HQ67595, HQ667596 and HQ667592 respectively. The nuc-LSU-rDNA sequences were successfully obtained from *Tulostoma dominguezziae* MLHC 24 (Holotype) as well as *Tulostoma* sp. (MLHC 200, MLHC 210) with GenBank accession numbers HQ667597, HQ667598 and HQ667599 respectively.

The aligned dataset of the ITS sequences consists of 14 taxa (TABLE I) with 730 characters. A total of 150 characters were excluded from the dataset due to ambiguous alignment. An additional 494 characters were parsimony uninformative. The remaining 86 characters were parsimony informative and used for MP analyses. The aligned dataset of the nuc-LSU-rDNA sequences consists of 10 taxa (TABLE I) with

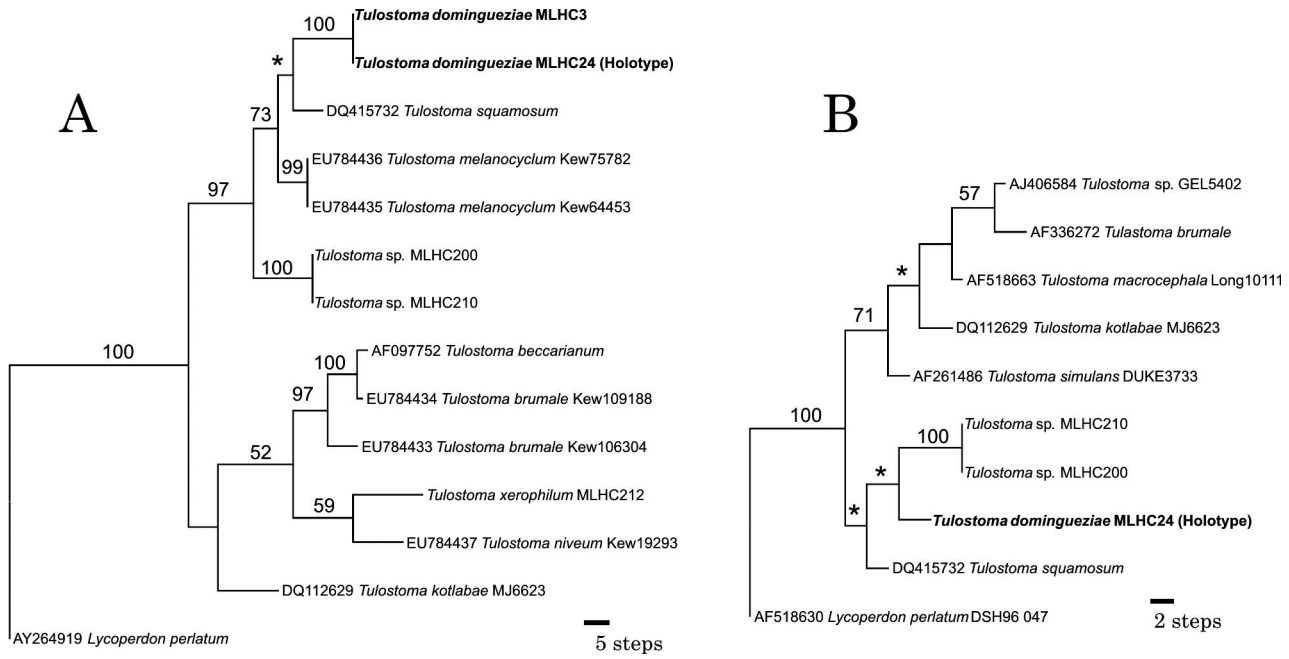


FIG. 20. One of the most parsimonious trees obtained from (A) ITS dataset, and (B) nuc-LSU-rDNA dataset. The GenBank accession number followed by taxon names and voucher numbers (if available) are shown. The numbers above branches indicate bootstrap support and asterisks indicate the nodes that were collapsed in strict consensus.

900 characters. A total of 22 characters were excluded from the dataset due to ambiguous alignment. An additional 852 characters were parsimony uninformative. The remaining 26 characters were parsimony informative and used for the analyses. We also used different taxa in the Agaricaeae (e.g. *Coprinus*, *Agaricus* etc.) as outgroup, but phylogenetic trees were almost identical (FIG. 20) (data not shown).

MP analyses of the ITS dataset produced two equally parsimonious trees with the tree length 184, CI 1.6630, HI 0.3370, RI 0.7406 and RC 0.4910. The topology of two trees differs slightly in terms of the position of *T. dominguezeiae*, but both trees showed that *T. dominguezeiae* is closely related to *T. melanocyclum* and *T. squamosum* (FIG. 20A). None of the available sequences from the GenBank matched those of *T. dominguezeiae*.

MP analyses of the nuc-LSU-rDNA dataset produced three equally parsimonious trees with the tree length 49, CI 0.6122, HI 0.3878, RI 0.6415 and RC 0.3928. The position of *T. dominguezeiae* was not strongly supported, but the results clearly showed that *T. dominguezeiae* is distinct from any other species tested (FIG. 20B). No sequences from *T. macalpineanum*, *T. dumeticola* and *T. exasperatum*, which possess a warty or verrucose exoperidium, are currently available from the database. Future study is necessary to collect these species and to assess their phylogenetic positions.

Although taxon sampling from genus *Tulostoma* is not extensive, newly generated sequences from this study, especially those from the holotype specimens, are useful in molecular identification of the species. We have retrieved all ITS and nuc-LSU-rDNA sequences that belong to *Tulostoma*, and none of them matched those of *T. dominguezeiae*. This is consistent with our morphological observations, which clearly demonstrated that *T. dominguezeiae* is a distinct and new species to science. Future study with more taxon and character sampling is necessary to determine the phylogenetic position of *T. dominguezeiae*.

KEY TO *TULOSTOMA* SPECIES WITH WARTY TO VERRUCOSE EXOPERIDIUM AND RELATED SPECIES

1. Mouth margin fibrillose . . . . . *T. exasperatum*
1. Mouth margin smooth 2
   2. Socket absent . . . . . *T. leprosum*
   2. Socket present 3
3. Exoperidium hyphal . . . . . *T. melanocyclum*
3. Exoperidium membranous, warty to verrucose . . . 4
   4. Stipe with scales arranged in rings . . . *T. matae*
   4. Stipe if scaly, scales not arranged in rings . . 5
5. Spores almost smooth . . . . . *T. macalpineanum*
5. Spores with conspicuous ornamentation 6
   6. Stipe contorted; socket with up to 6 dentate membranes . . . . . *T. dominguezeiae*
   6. Stipe straight; socket with 1 dentate membrane 7

TABLE I. Strains used in this study

Collection	Specimen number	Identified by	Country of origin	Herbarium	GenBank number (ITS)	GenBank number (LSU)
T. beccarianum	1663	N/A	Spain	BCC-MPM	AF097752	
T. brumale	109188	T Boniface	UK	K	EU784434	
T. brumale	106304	BM Spooner	UK	K	EU784433	
T. dominguezieae	24	MLHC	Argentina	CORD	HQ667594	HQ667597
T. dominguezieae	3	MLHC	Argentina	CORD	HQ667593	
T. kotlabae	6623	M Jeppson	Hungary	GB	DQ112629	
T. macrocephala	10111	Long	N/A	FH	AF518663	
T. melanocyclum	64453	PJ Roberts	UK	K	EU784435	
T. melanocyclum	75782	BM Spooner	UK	K	EU784436	
T. niveum	19293	CEK Scouller	UK	K	EU784437	
T. simulans	3733	A Odom	USA	DUKE	AF261486	
T. sp.	200	MLHC	Argentina	CORD	HQ67595	HQ667598
T. sp.	210	MLHC	Argentina	CORD	HQ667596	HQ667599
T. squamosum	1300	Mrazek	Austria	GB	DQ415732	
T. xerophylum	212	MLHC	Argentina	CORD	HQ667592	

7. Spore ornamentation made of conical spines, under SEM, made of 6–9 columns united at the apex, up to 2 µm high never anastomosing . . . . . *T. pusillum*
7. Spores ornamentation not as above 8
   8. Spores reticulate . . . . . *T. subsquamosum*
   8. Spores echinulate 9
9. Socket with entire membrane. Growing in bare soil at altitude exceeding 3600 m (South America). Spores (5.5–)6.8–7.8(–8.5) µm diam . . . *T. dennisii*
9. Socket with irregularly torn membrane. Growing at lower altitude. Spores slightly smaller 10
   10. Socket conspicuous. Spores globose-subglobose, 3.4–6.5 × 4.7–5.8 µm. Under SEM ornamentation made up of spines and a few fused and forming short crests. Growing in calcareous soil with vegetation. . . *T. squamosum*
   10. Socket inconspicuous. Spores globose 5.4–7.2 µm. Under SEM ornamentation made of spines, many anastomosing forming an incomplete reticulum. Growing at low elevations in forest soil among litter and humus . . . . . *T. dumeticola*

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