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RESEARCH ARTICLE

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Talaromyces halophytorum sp. nov. Isolated from Roots of *Limonium tetragonum* in Korea

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ABSTRACT

Talaromyces halophytorum sp. nov. was isolated from the roots of halophyte *Limonium tetragonum* collected from Seocheon-gun, Korea in November 2015. It showed a slow growth on yeast extract sucrose agar at 25 °C, no growth at 4 °C or 37 °C and produced smooth-walled and globose to sub-globose conidia. *T. halophytorum* is phylogenetically distinct from the other reported *Talaromyces* species of section *Trachyspermi* based on multi-locus sequence typing results using partial fragments of β-tubulin, calmodulin, ITS, and RNA polymerase II genes.

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1. Introduction

The genus *Talaromyces* was established by Benjamin (1955) as a name for a number of *Penicillium* species that could produce a sexual state. The genus was characterized by producing soft ascocarps with cleistothecial walls that have multiple layers of interwoven hyphae and typically yellow ascomata with ovate to globose asci with spiny ascospores [1–3]. Based on phenotypic, extrolite, phylogenetic data and the concept of one fungus one name, Samson et al. [4] transferred the majority of accepted species of *Penicillium* subg. *Biverticillium* to *Talaromyces*. In 2014, a total of 88 species were accepted in the monograph of the genus. These species were classified in seven well-defined sections, namely *Talaromyces*, *Bacillispori*, *Helici*, *Purpurei*, *Trachyspermi*, *Subinflati*, and *Islandici* [3]. Subsequent to the monograph of Yilmaz et al. [3], 73 new *Talaromyces* species such as *T. amyrossmania*, *T. heiheensis*, *T. minnesotensis*, *T. rubrifaciens*, and many more have been described from all over the world [5–10].

Talaromyces species are thoroughly associated with human life since they could be used as potential anti-cancer [11], anti-fungal [12], and anti-*Trypanosoma* [13] agents. *Talaromyces* species have also received considerable attention as biotechnological resources such as purpactins (produce by *T. purpurogenus* as inhibitors of acyl–coenzyme A), anti-influenza virus polyketides [14,15], antibiotics [12], food dyes [16], and exoenzymes such as dextranases, cellulases, glucoamylases, and chitinases [17–20]. Other *Talaromyces* species, like *T. marneffei*, are isolated from clinical specimens [21] and can cause fatal mycoses in immunocompromised individuals [22].

Limonium tetragonum (Thunb.) A. A. Bullock (Plumbaginaceae) is a species of salt-tolerant plants that grows in saline environments such as coastal sand dunes, salt marsh, and muddy seashores throughout the western coastal area of Korea [23]. Roots and leaves of this plant are widely used as edible vegetables in Korea. It has been reported that it has antioxidant and anti-cancer properties due to the presence of numerous bioactive materials [24,25].

In the present study, we report a new *Talaromyces* species in section *Trachyspermi* isolated from *L. tetragonum*.

2. Materials and methods

2.1. Collection, strain isolation, and preservation

L. tetragonum plants were collected from the coast of Seocheon-gun, Chungcheongnam-do, Korea in November 2015. Samples were transported to the

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laboratory and processed for isolation of endophytic fungi within 24 h. First, the plant material was rinsed with tap water to remove dust and debris. The stem and root were then cut into small pieces with a sterilized blade under aseptic conditions. Five tissue segments were randomly selected from each part. These plant tissues were surface-sterilized by consecutive immersions in 70% ethanol for 30 s and in 1% sodium hypochlorite (NaOCl; Duksan, Ansan, Korea) for 1 min. These samples were then rinsed with sterile water three times and allowed to surface dry on filter paper. The sets of five tissue segments from stem and root were placed on potato dextrose agar (PDA; Difco, Sparks, MD, USA) plate and incubated at 25 °C. As the fungus grew from the tissue segment, newly emerging hyphal tips were transferred onto new PDA plates. These plates were incubated at 25 °C to obtain single hyphae isolate. Pure cultures of fungal strains were preserved on PDA slant at 4 °C. A representative strain (WLT07) was deposited in the Korean Agricultural Culture Collection, National Institute of Agricultural Science, Rural Development Administration, Wanju, South Korea (KACC 48127) to be used for further studies.

2.2. Morphological analysis

Strain WLT07 was morphologically studied on different media under different growth conditions. The strain was inoculated onto malt extract agar (MEA; Oxoid, Hampshire, UK), Czapek yeast extract agar (CYA; Difco), yeast extract sucrose agar (YES), oatmeal agar (OA; Difco), and creatine sucrose agar (CREA) at three points on 90-mm Petri dishes and incubated at 25 °C in the dark for 7 days. All media were prepared as described by Visagie et al. [26]. Additional CYA plates were incubated at 4 °C and 37 °C for 7 days in the dark. After incubation, diameters of colonies on each medium were measured. The density of sporulation, obverse and reverse colony colors, and the production of soluble pigments were noted. Fungal colonies were photographed with a Canon EOS 400 D camera (Tokyo, Japan). Morphological characterization was performed by observing the slides prepared from MEA using light microscopy (DE/Axio Imager.A1, Carl Zeiss, Göttingen, Germany). Lactic acid was used as a mounting fluid and a drop of ethanol was added to remove excess conidia. Specimen images were acquired using AxioVision LE64 software (Carl Zeiss, Oberkochen, Germany). Figure plates were prepared with Photoshop CS2 (Adobe, San Jose, CA, USA).

2.3. Multi-gene sequencing and phylogenetic analysis

To extract genomic DNA, strain WLT07 was grown in malt extract broth medium (MEB; Oxoid, Hampshire, UK) on an orbital shaker for 2–4 days at 25 °C. Its fungal mycelia were harvested by filtration and transferred to sterile 1.5 mL tubes. These samples were frozen at −70 °C, lyophilized, and finely ground. DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The internal transcribed spacer (ITS) region of the ribosomal DNA was amplified using ITS1 and ITS4 primers [27]. In addition, three nuclear protein genes were amplified (partial β-tubulin (*benA*) gene was amplified using Bt2a and Bt2b [28]; partial DNA-dependent RNA polymerase II second largest subunit (*Rpb2*) gene was amplified using RPB2-5F and RPB2-7CR [29]; and partial calmodulin (*CaM*) gene was amplified using CMD5 and CMD6 [30]). PCRs were performed in 25 µL reaction tubes containing 2.0 µL DNA template, 0.5 µL of each forward and reverse primers (10 µmol L^{-1}), 0.5 µL *Taq* DNA polymerase (Bioneer, Daejeon, Korea), 0.5 µL of each dNTP, 2.5 µL 10× PCR reaction buffer, and 18.5 µL of sterile double-distilled water. Thermal cycling conditions were as follows: initial denaturation at 94 °C for 3 min; 35 cycles of 94 °C for 40 s, 54 °C for ITS, 58 °C for *benA*, 55 °C for *CaM*, or 48 °C for *RPB2* for 60 s, and 72 °C for 2 min; and a final elongation step of 72 °C for 10 min. PCR products were purified and sequenced by Macrogen (Seoul, Korea). Nucleotide sequences obtained were searched using BLASTn available in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) to obtain the most likely taxonomic designations for the strain (Table 1). Evolutionary matrices for the maximum likelihood, neighbor-joining, and maximum parsimony were constructed using Kimura's two-parameter model [31]. Phylogenetic tree topology was inferred by the maximum likelihood, neighbor-joining, and maximum-parsimony method using MEGA6 with bootstrap values based on 1000 replications [32].

3. Results

3.1. Morphological characterization

Photomicrographs of morphological structures of the WLT07 strain are shown in Figure 1. Detailed morphological characters are described in the Taxonomy section (Section 3.3).

3.2. Phylogenetic analysis

Sizes of PCR amplicons were 551 bp for ITS, 423 bp for *BenA*, 479 bp for *CaM*, and 1088 bp for *RPB2*.

Table 1. Accession numbers of fungal strains used for the phylogenetic analysis.

Species	Collection no.	GenBank accession no.			
		ITS	BenA	CaM	RPB2
Talaromyces atroroseus	CBS 133442^T	KF114747	KF114789	KJ775418	KM023288
T. albobiverticillius	CBS 133440^T	HQ605705	KF114778	KJ885258	KM023310
T. convolutus	CBS 100537^T	JN899330	KF114773	–	JN121414
T. diversus	CBS 320.48^T	KJ865740	KJ865723	KJ885268	KM023285
T. erythromellis	CBS 644.80^T	JN899383	HQ156945	KJ885270	KM023290
T. halophytorum	**WLT07**	**MH725786**	**MH729367**	**MK111426**	**MK111427**
T. solicola	CBS 133445^T	FJ160264	GU385731	KJ885279	KM023295
T. minioluteus	CBS 642.68^T	JN899346	KF114799	KJ885273	JF417443
T. ucrainicus	CBS 162.67^T	JN899394	KF114771	KJ885282	KM023289
T. assiutensis	CBS 147.78^T	JN899323	KJ865720	KJ885260	KM023305
T. trachyspermus	CBS 373.48^T	JN899354	KF114803	KJ885281	JF417432
T. bacillisporus	CBS 296.48^T	KM066182	AY753368	KJ885262	JF417425
T. proteolyticus	CBS303.67^T	JN899387	KJ865729	KJ885276	KM023301
T. emodensis	CBS 100536^T	JN899337	KJ865724	KJ885269	JN121552
T. mimosinus	CBS 659.80^T	JN899338	KJ865726	KJ885272	–
T. palmae	CBS 442.88^T	JN899396	HQ156947	KJ885291	KM023300
T. subinflatus	CBS 652.95^T	JN899397	KJ865737	KJ885280	KM023308
Trichocoma paradoxa	CBS 788.83	JN899398	KF984556	KF984670	JN121550

The isolated strains are indicated in bold of this study. T: Type strain.

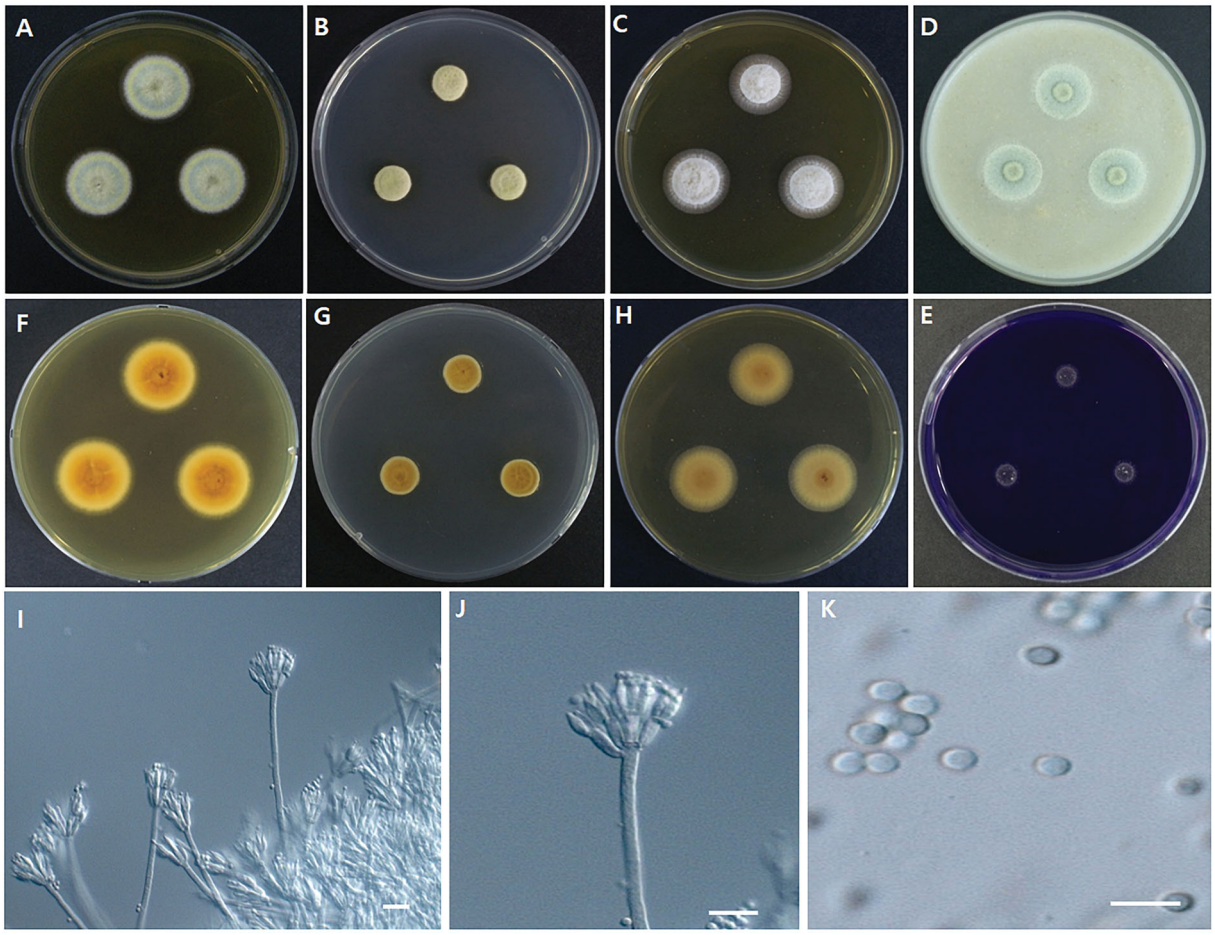

Figure 1. Morphological characteristics of *Talaromyces halophytorum* sp. nov., WLT07. (A,F) Colony on MEA; (B,G) Colony on CYA; (C, H) Colony on YES; (D) Colony on OA; (E) Colony on CREA; (I–J) Conidiophores (scale bar = 10 μm); (K) Conidia (scale bar = 10 μm).

Sequences obtained from strain WLT07 were deposited in the NCBI database (GenBank accession numbers: MH725786 for ITS, MH729367 for *BenA*, MK111426 for *CaM*, and MK111427 for *RPB2*). Phylogenetic analysis based on the maximum-likelihood methods of combined ITS, *BenA*, *CaM*, and *RPB2* sequences showed that strain WLT07 is closely related with type strains of *T. solicola* CBS 133445 (96.3% homology), *T. albobiverticillius* CBS 133440 (93.5%), and *T. erythromellis* CBS 644.80 (93.4%). Neighbor-joining and maximum-parsimony phylograms were also constructed to determine the exact taxonomic position of the strain and the bootstrap values are indicated at the nodes in the maximum-likelihood phylogenetic tree. Filled circles indicated that corresponding nodes were also

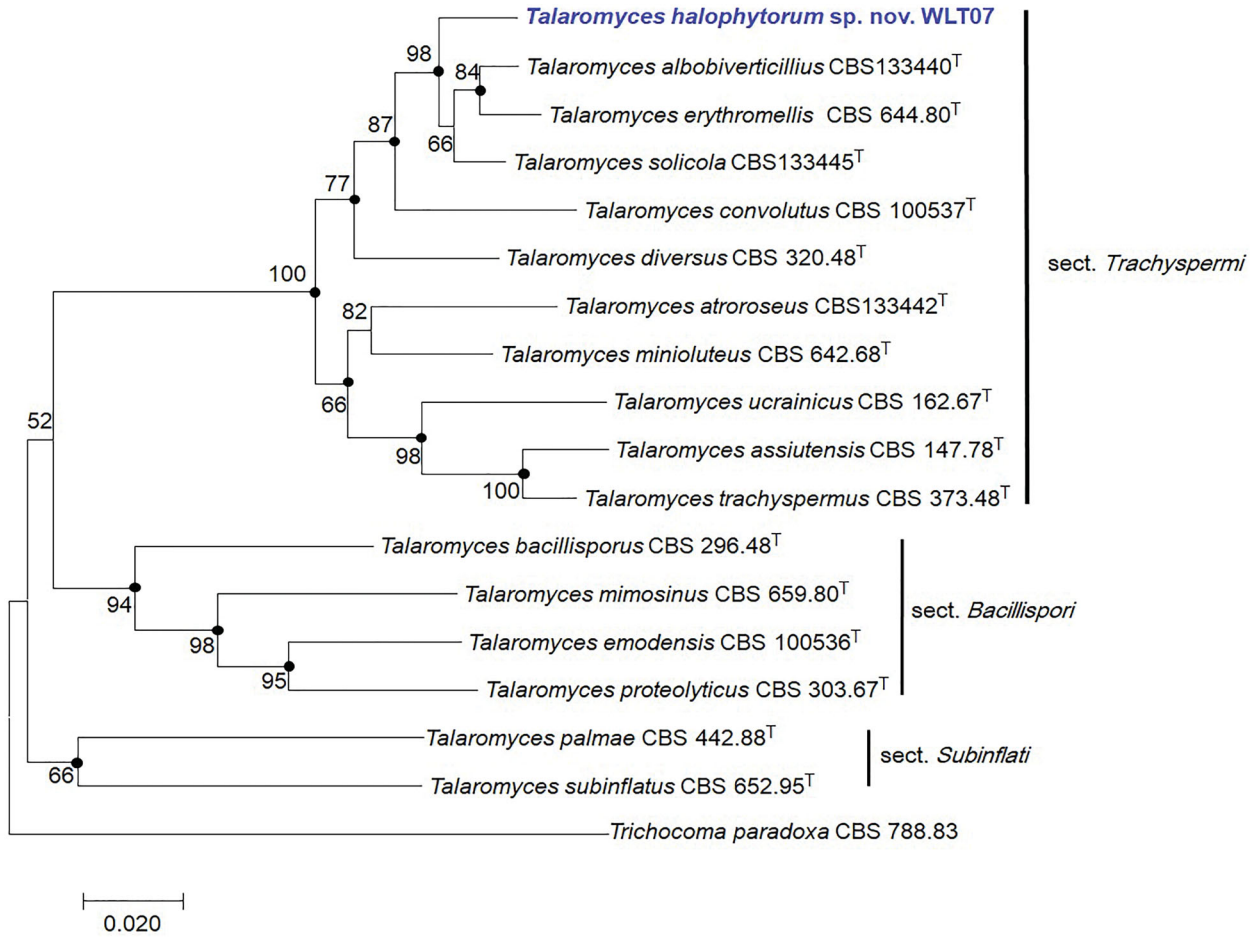

Figure 2. Maximum-likelihood phylogenetic tree based on combined ITS, *BenA*, *CaM*, and *RPB2* genes of *Talaromyces section Trachyspermi* species including *Talaromyces halophytorum* sp. nov., WLT07. Filled circles indicate that corresponding nodes are also recovered in the trees generated with the maximum-parsimony and neighbor joining algorithms. *Trichocoma paradoxa* was included as an outgroup. Bootstrap analysis was performed with 1000 replications. T indicates the type strain of the species. Bar, 0.02 substitutions per nucleotide position.

recovered in trees generated with the neighbor-joining and maximum parsimony algorithms (Figure 2). The phylogenetic tree revealed that the phylogenetic position of WLT07 was clearly separated from *T. solicola*, *T. albobiverticillius*, and *T. erythromellis*. Thus, WLT07 was phylogenetically distinct from other species of *Talaromyces*.

3.3. Taxonomy

Talaromyces halophytorum, Y. H. You and S. B. Hong, sp. nov. (Figure 1).

Mycobank: MB830295.

Etymology: ha.lo.phy.to'rum, N.L. gen. pl. n. halophytorum of halophytes.

In: *Talaromyces* section *Trachyspermi.*

Typus: KACC 48127 (NIBRFGC 000501933).

Gene sequence: ITS= MH725786; *BenA*= MH729367; *CaM*=MK111426; *RPB2* = MK111427.

Colony diam., 7 d (mm): MEA 24–25; CYA 12–13; CYA 4 °C and 37 °C No growth; YES 18–20; OA 15–6; CREA 3–5.

Colony characteristics: MEA, 25 °C, 7 days: colony greenish gray in color, reverse brownish orange, raised in center, concentric, margins narrow (1–2 mm), low, entire plane; texture floccose; sporulation dense, conidia *en masse* grayish green; exudates clear, sometimes orange; soluble pigment absent (Figure 1(A,F)). CYA, 25 °C, 7 days: colony gray in color, reverse brownish orange to yellowish brown, raised at margins, occasionally cleaved (or cracked) in the middle, texture mainly velvety, occasionally floccose; sporulation dense, conidia *en mass* grayish green; soluble pigment, and exudate absent (Figure 1(B,G)). YES, 25 °C, 7 days: colony white in color, backside light orange, flat, slightly concentrically sulcate, margins entire; texture floccose; sporulation sparse; soluble pigment and exudate absent (Figure 1(C,H)). OA, 25 °C, 7 days: colony greenish to green, raised at center, texture velvety, especially near center; sporulation moderately to dense; soluble pigment and exudates absent (Figure 1(D)). CREA, 25 °C, 7 days: very weak growth, acid production absent (Figure 1(E)).

Micromorphology: Conidiophores biverticillate, 70–180 × 3–4 µm. Subterminal branches absent. Stipes smooth-walled. Phialides acerose, 3–5 per metula, and 6.5–11 × 2.5–3 µm in size. Metulae

arranged in verticils of 5–8, and 6.5–11 × 2.5–4 µm in size (Figure 1(I,J)). Conidia globose to subglobose, 2.5–3.5 × 2–3 µm in diameter, smooth-walled (Figure 1(K)). Ascomata absent.

Distinguishing characters: *Talaromyces halophytorum* is characterized by its slow growth on CYA and no growth on CYA at 4 °C or 37 °C. Conidiophores are biverticillate and it produces smooth-walled globose to sub-globose conidia. Phylogenetically, it is closely related to *T. silicola*, *T. erythromellis*, and *T. albobiverticillius*. However, it is differentiated from these three species in morphology. *T. erythromellis* produces symmetrical subterminal branches and smooth-walled sub-globose to ellipsoidal conidia, whereas *T. solicola* and *T. albobiverticillius* produce rough-walled globose to sub-globose shaped conidia.

4. Discussion

A phylogenetic approach based on multiple genes [33] (ITS, *BenA*, *CaM*, and *RPB2*) was applied to study the relationship of *T. halophytorum* in *Talaromyces* section *Trachyspermi*. Our results inferred from the phylogenetic analysis of combined ITS, *BenA, CaM*, and *RPB2* sequences indicated that *T. albobiverticillius*, *T. erythromellis*, and *T. solicola* formed a group [3,16]. Phylogenetic trees revealed that strain WLT07 is distinct from other known *Talaromyces* species. This result is confirmed by results of neighbor-joining, maximum parsimony, and maximum-likelihood phylogenetic trees (Figure 2). Yaguchi et al. [34] introduced *Talaromyces* section *Trachyspermi* (as "*trachyspermus*") based on ubiquinone systems that overrode the traditional morphology-based classification of *Talaromyces*. Section *Trachyspermi* was established for species that grew restrictedly on CYA, YES, and DG18, slightly faster on MEA, and poorly on CREA. Conidiophores are biverticillate and ascomata, if present, have a cream white or yellow color [3]. *T. halophytorum* strain WLT07 in this group is supported by similarities in its morphological characters including restricted growth on CYA, YES, slightly faster growth on MEA, poor growth on CREA, biverticillated conidiophores, smooth-walled conidia, and globose to sub-globose shape (Figure 1). Despite these similarities, *T. halophytorum* could be distinguished from *T. solicola*, *T. erythromellis*, and *T. albobiverticillius* by distinctive phenotypic characters such as colors on CYA, CYA soluble pigment, conidial ornamentation, and shape. The reverse side color on CYA of *T. halophytorum* was brownish orange to yellowish brown, whereas that of *T. solicola* was reddish brown to dark brown, that of *T. erythromellis* was dark red to brownish red, and that of *T. albobiverticillius* was grayish red-brown. Only *T. albobiverticillius* produced red pigments on CYA. Another notable feature of *T. halophytorum* was that it produced biverticillated conidiophores and smooth-walled globose to sub-globose conidia, whereas *T. erythromellis* produced symmetrical subterminal branches and smooth-walled sub-globose to ellipsoidal conidia and *T. solicola* and *T. albobiverticillius* produced rough conidia with globose to sub-globose shape [3,16,35]. In conclusion, our data show that this strain represents a new species of *Talaromyces* in section *Trachyspermi*. *Talaromyces halophytorum* sp. nov. is proposed as its name.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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