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Ahmadea dalanensis gen. and sp. nov., an edible truffle from Pakistan

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Abstract

Ahmadea, a monotypic truffle genus is described morphologically and its position in the family Pezizaceae (Pezizales, Ascomycota) is inferred from the phylogenetic analyses of DNA sequences obtained from the large subunit nuclear ribosomal RNA gene (LSU). Its type species, *A. dalanensis*, is found in arid and semi-arid regions of Punjab, Pakistan, often occurring in *Sorghum vulgare* crop fields where it has been known for its edibility for ages. Relationships of *Ahmadea* with the related truffle genera *Stouffera* Kovács & Trappe, *Temperantia* K. Hansen, Healy & Kovács, *Hydnobolites* Tul. & C. Tul. and *Delastria* Tul. & C. Tul. are discussed in this paper.

Key words – Ascomycota – ITS paralogues – LSU – Pezizaceae – Pezizales – Sorghum – Taxonomy

Introduction

Truffles are fungi that produce enclosed fruiting bodies underground or slightly above ground and lack an active spore discharge mechanism (Laessoe & Hansen 2007). The taxonomy of truffles has been historically problematic and remains challenging in the molecular era. Most ascomycetous truffles were initially believed to belong to Tuberales (Korf 1973) but have since been placed in Pezizales (Trappe 1979, Laessoe & Hansen 2007). Molecular phylogenetic studies by O'Donnell et al. (1997), Hansen et al. (2001) and others have shown that some hypogeous fungi are more closely related to epigeous fungi within Pezizales than to other hypogeous members of the Order. Hansen (2006) segregated the ascomycetous truffles into six families within the Order Pezizales: Pyronemataceae, Helvellaceae, Glaziellaceae, Discinaceae-Morchellaceae, Pezizaceae and Tuberaceae. The latter comprises highly prized truffles such as *Tuber melanosporum* Vittad. and allied species. Within Pezizaceae, Hansen (2006) estimated that there were at least 15 independent events of above ground fungi evolving to the hypogeous habit. More recently, Kovács et al. (2011) identified and described two new genera of truffles within Pezizaceae: *Temperantia*, and *Stouffera*.

Truffles have been documented and studied from many regions of the world, including Kalahari Africa (Trappe et al. 2008), Australia (Francis & Bougher 2002, Lebel & Castellano 1999), the Algerian Sahara (Bradai et al. 2014), Mexico (Gomez-Reyes et al. 2017), North America (Trappe & Castellano 1991, Izzo et al. 2005, Laessoe & Hansen 2007) and China (Li et al. 2019). In the arid regions of Pakistan, some truffles are known for their edibility but scientific studies are lacking for this region of the world and their taxonomy is unclear. The aim of this study was to identify truffle specimens collected from two different locations in semi-arid and arid regions of Pakistan, place them phylogenetically, and name them.

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Materials & Methods

Collection sites

Sampling was conducted at three different localities in semi-arid and arid regions of Punjab, Pakistan. Four samples were collected from three different localities. One sample was collected from sorghum fields in Dalana, Dera Ghazi Khan (30.0629° N, 70.4484° E) in September 2017. Two samples were collected near shrubs at the Jhok Reserve forest (31.4343° N, 74.1387° E), Lahore, in August 2018 and one sample was collected from the Shahdara Reserve Forest (31.6286° N, 74.3647° E), Lahore, in August 2018. Collected specimens were photographed and tagged. The specimens were then dried at 35 to 40ºC under a fan heater and kept in zip-lock bags. Dried specimens were deposited in the MS Zahoor Herbarium, Department of Botany, University of the Punjab, Lahore, Pakistan, and in the Fungarium of the Royal Ontario Museum (TRTC), Toronto, Ontario, Canada.

Morphological examination

Specimens were photographed and described macromorphologically. Microscopic slide preparations were made by placing dried gleba tissues in 5% aqueous KOH and in 1% Congo red in ammonia (w/v). Ascus size and shape, and ascospore number, size and shape were recorded. At least 20 asci and ascospores were measured. The Q value was calculated as length/width ratio, avQ being the mean length/width ratio of all ascospores. Glebal portions were sent to the Centralized Resource Laboratory at the University of Peshawar, Pakistan, to obtain SEM images of the ascospores.

DNA extraction, PCR, and sequencing

DNA was extracted from small glebal portions of dried specimens using the CTAB method (Bruns 1995). ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990) primers were used for PCR amplification and sequencing of the internal transcribed spacer region of the nuclear rRNA genes (ITS). A portion of the 5' region of the nuclear large subunit rRNA genes (LSU) was amplified and sequenced using the primer pair LR0R/LR5 (Vilgalys & Hester 1990). Polymerase chain reactions were performed in 20 µL with PCR 2X master mix (Wizsolutions, New Jersey, USA), 5 µL dd water, 1 µL $MgCl_2$ and 1 µL of each primer. Thermo-cycling conditions were: initial denaturation at 94°C for 5 min, then 35 cycles of 94°C for 1 min, 53°C for 1 min, 72°C for 1 min, and final extension at 72°C for 18 min. PCR products were visualized in 1% agarose gel stained with 3 µl ethidium bromide. Sequencing of the amplified products was performed by TsingKe Biological Technology Company (Beijing, China).

DNA sequences analyses

Consensus sequences were edited in Sequencher 3.0 (Genes Codes Corp.). Similar sequences were retrieved from BLAST searches (Altschul et al. 1990) in the NCBI database (https:www.ncbi.nlm.nih.gov), and the top 100 hits were downloaded. We also retrieved from the NCBI database other relevant LSU sequences identified in the study conducted by Kovács et al. (2011), including two sequences of *Ascobolus* Pers. for rooting purpose. Sequence alignments were conducted in MUSCLE 3.6 (Edgar 2004) from https:www.ebi.ac.uk/Tools/msa/muscle/, then visualized and manually adjusted in AliView (Larsson 2014). Several preliminary sequence analyses were conducted in MEGA7 (Kumar et al. 2016) in order to select the best possible set of sequences for our study. Our final dataset consisted of 79 aligned sequences. The best-fit maximum-likelihood (ML) model was estimated in IQ Modelfinder (Kalyaanamoorthy et al. 2017) and implemented in IQ-tree (Nguyen et al. 2015) from the web server at http://iqtree.cibiv.univie.ac.at/. The best-fit ML model was determined to be GTR+F+I+G4. Statistical support for branches was calculated from 1000 bootstrap replicates. Phylogenetic trees were visualized in FigTree V.1.4.0 (Rambaut 2012) and edited in Adobe illustrator CS5 and Adobe Photoshop 7.0 for presentation.

Results

Phylogenetic analyses

The final results of our phylogenetic analyses are presented in Fig. 1. Two clades were clearly distinguishable. One clade (99% bootstrap support) consisted primarily of *Peziza* species including its type species (*Peziza phyllogena* Cooke) as well as several truffle genera in derived positions (*Hydnotryopsis* Gilkey, *Mattirolomyces* E. Fisch., *Elderia* McLennan, *Ruhlandiella* Henn., *Mycoclelandia* Trappe & GW Beaton and *Terfezia* Tul & C. Tul). The second clade (98% bootstrap support) consists of three of our collections (presented below as *Ahmadea dalanensis* gen. and sp. nov.) along with the truffle genera *Delastria*, *Hydnobolites*, *Stouffera* and *Temperantia*, with typical Pezizaceae cup fungi of *Marcelleina* Brumm., Korf & Rifai and *Peziza gerardii* Cooke as the sister group. In Fig. 1, *Ahmadea dalanensis* sp. nov. clusters with *Stouffera longii* (Gilkey) Kovács & Trappe with low bootstrap support (68%). However, when that second clade was analyzed independently using *Peziza gerardii* and *Marcelleina* spp. as outgroups, *Ahmadea* gen. nov. clusters with *Temperantia tiffanyae* K. Hansen, Healy & Kovács with still relatively weak 75% bootstrap support (data not shown). Fig. 1 also shows that our sample, labeled SRF3, corresponds to *Mattirolomyces spinosus* (Harkn.) Kovács, Trappe & Alsheikh (Kovács et al. 2011).

BLAST searches of ITS sequences from our *A. dalanensis* samples (two specimen) retrieved sequences from *Hydnobolites* and *Delastria*, but revealed extremely low coverage (22-31%) and very poor E-values (4e-59 to 1e-55). Additionally, matches were only in the 5.8S gene region and showed only 94% similarity.

Taxonomy

***Ahmadea* Aman, Khalid & Moncalvo**, gen. nov.

Index Fungorum number: IF557795; Facesoffungi number: FoF09164

Etymology – Ahmadea (Latin), in honour and memory of Dr. Sultan Ahmad (1910-1983): His work was instrumental for the documentation of fungi in Pakistan and an inspiration for the development of mycology in Pakistan.

Diagnosis – Hypogeous ascomata, irregular in shape, thin excipulum, solid gleba lacking paraphyses, asci with eight spores (sometimes less), ascospores globose, ornamented with sharp spines with a broad base and blunt ends.

Type species – *Ahmadea dalanensis* Aman & Khalid, sp. nov.

Notes – We are introducing a new genus in the family Pezizaceae. It is so far monospecific and based on a truffle, *Ahmadea dalanensis*, which is described below. The proposal for this new genus is based upon combined evidence derived from the morphology and LSU sequence phylogeny.

***Ahmadea dalanensis* Aman & Khalid**, sp. nov. Figs 2, 3

Index Fungorum number: IF557796; Facesoffungi number: FoF09163

Etymology – The specific epithet “dalanensis” refers to the locality from where our first specimen of the new species was collected: Dalana, Dera Ghazi Khan, Punjab, Pakistan.

Holotype – PAKISTAN, Punjab Province, Lahore, Jhok Reserve Forest, on the ground near *Acacia nilotica* Delile, 18 August 2018, N. Aman, JRF10 (LAH36405, TRTC176230).

Ascocarp – hypogeous, white, smooth, irregular, covered with soil debris, 1.5 to 5 cm in diameter, *Gleba* consists of sterile, whitish, meandering veins, irregularly spread and surroundings fertile cream, yellow part containing ascus and ascospores. *Asci* globose to subglobose. *Asci* size 77-91 x 61-81µm hyaline to yellow in Melzers reagent.

Ascospores globose, 22.052 x 18.136 µm in diameter, Q = 1.21, ornamented, spiny with a broad base and blunt ends.

Specimen examined – PAKISTAN, Punjab Province, Lahore, Jhok Reserve Forest, on the ground near *Acacia nilotica* Delile, 18 August 2018, N. Aman, JRF10 (holotype LAH36405,

isotype TRTC176230); Punjab Province, Lahore, Jhok Reserve Forest, on the ground near *Acacia nilotica*, 18 August 2018, N. Aman JRF7 (LAH36404, TRTC176231); Dalana, Dera Ghazi Khan, in *Sorghum vulgare* L. Pers. field, 14 September 2017, N. Aman, TF7 (LAH36406, TRTC176232).

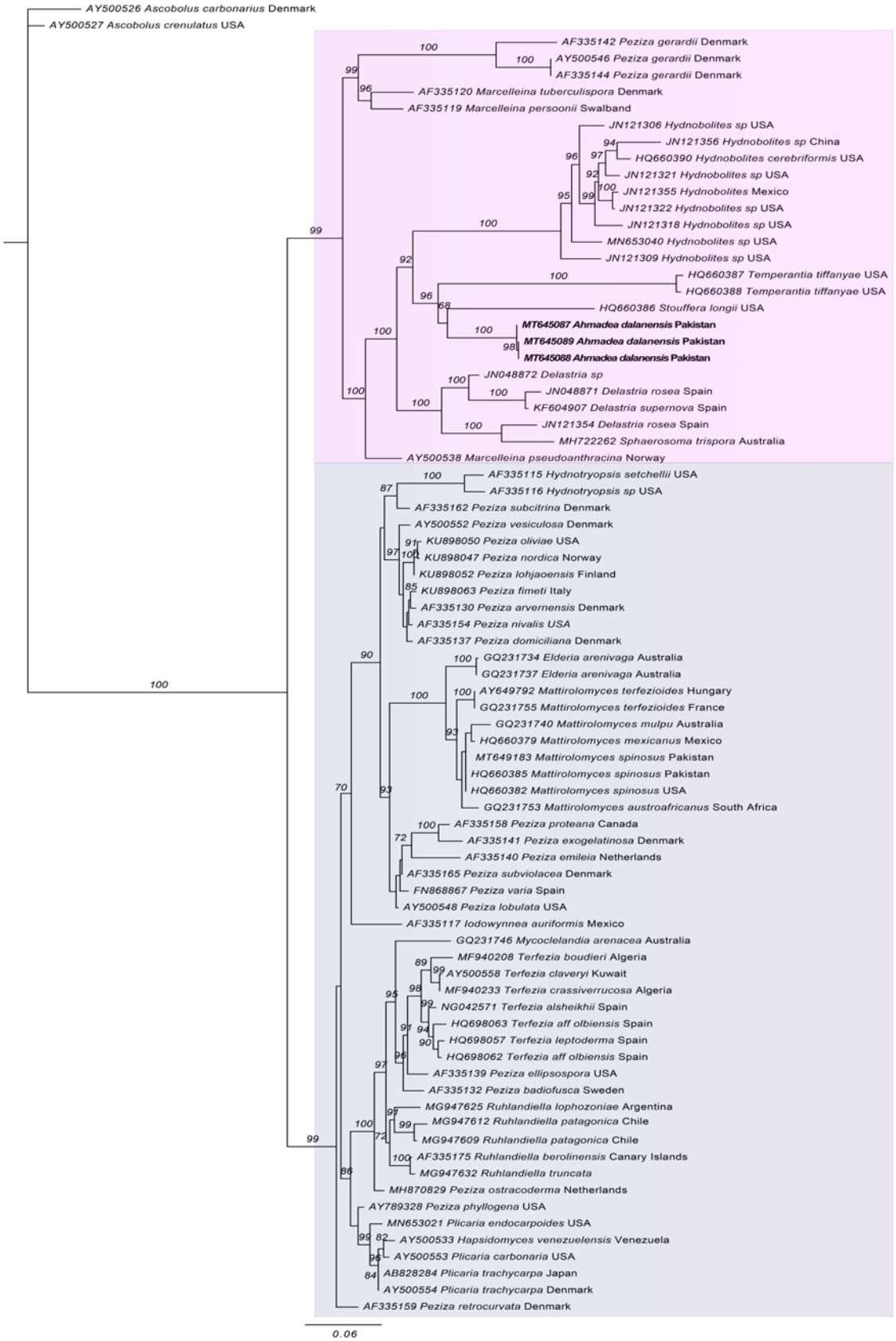

Fig. 1 – Maximum-likelihood tree based on LSU sequences showing the position of *Ahmadea dalanensis* gen. and sp. nov. within Pezizaceae showing two clades. *Ascobolus carbonarius* and *A.*

crenulatus serving as an outgroup to the root the tree. Bootstrap values below 60% are not shown. The taxa shown in boldface were collected and examined in the current research project.

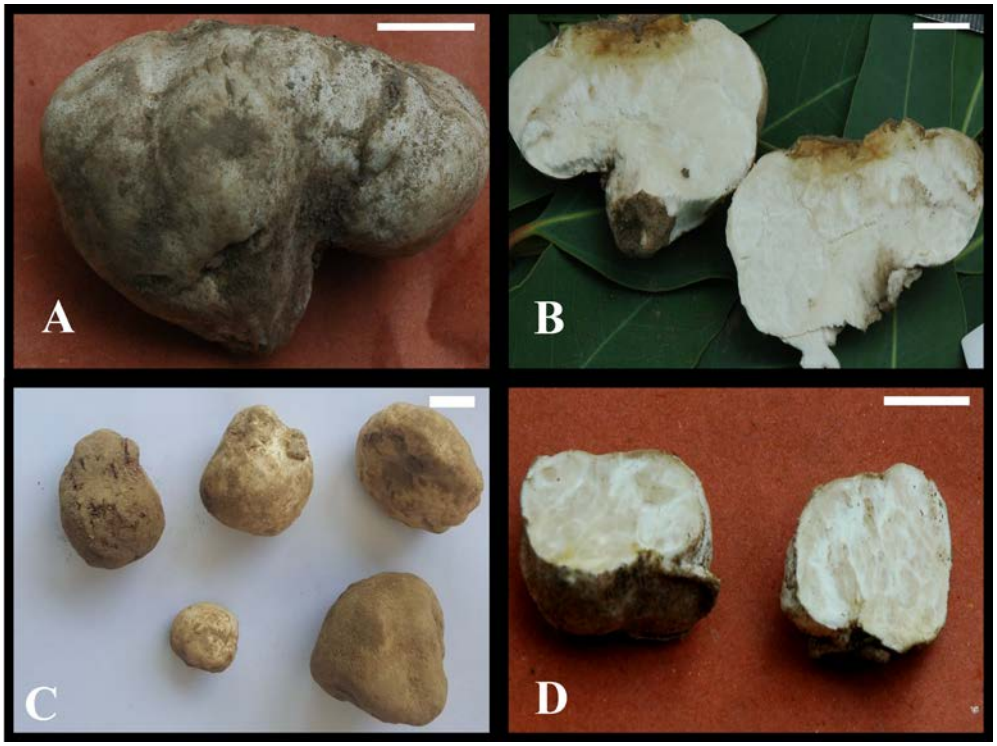

Fig. 2 – *Ahmadea dalanensis*: A Ascomata of Holotype (JRF10). B Gleba of Holotype (JRF10). C Fruiting bodies of paratype (TF7) showing different sizes. D Gleba of Paratype (JRF7). Scale bars: A–D = 1 cm.

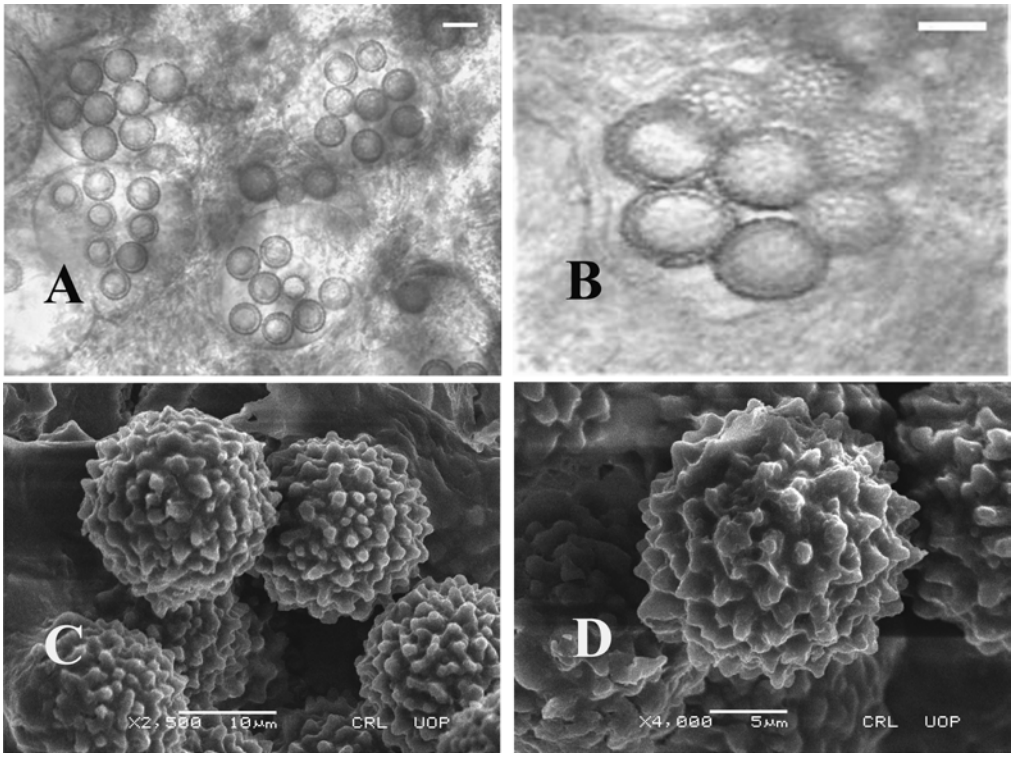

Fig. 3 – A Asci showing 8 ascospores (Holotype: JRF10). B Ascospores (Holotype: JRF10). C–D SEM images of ascospores showing ornamentation (Holotype: JRF10). Scale bars: A–B = 20 µm, C = 10 µm, D = 5 µm.

Table 1 Genbank accession numbers and geographic origin of the taxa used in the phylogenetic analysis that produced Fig. 1

Species name	**Geographic Origin**	**GenBank Accession number**
Ahmadea dalanensis (Holotype)	Pakistan	MT645087
Ahmadea dalanensis	Pakistan	MT645088
Ahmadea dalanensis	Pakistan	MT645089
Ascobolus carbonarius	Denmark	AY500526
Ascobolus crenulatus	USA	AY500527
Delastria rosea	Spain	JN121354
Delastria rosea	Spain	JN048871
Delastria sp.		JN048872
Delastria supernova	Spain	KF604907
Elderia arenivaga	Australia	GQ231734
Elderia arenivaga	Australia	GQ231737
Hapsidomyces venezuelensis	Venezuela	AY500533
Hydnobolites cerebriformes	USA	HQ660390
Hydnobolites sp.	USA	JN121306
Hydnobolites sp.	USA	JN121309
Hydnobolites sp.	USA	MN653040
Hydnobolites sp.	USA	JN121318
Hydnobolites sp.	USA	JN121321
Hydnobolites sp.	USA	JN121322
Hydnobolites sp.	Mexico	JN121355
Hydnobolites sp.	China	JN121356
Hydnotryopsis setchellii	USA	AF335115
Hydnotryopsis sp.	USA	AF335116
Iodowynnea auriformis	Mexico	AF335117
Marcelleina pseudoanthracina	Norway	AY500538
Marcelleina personii	Swalband	AF335119
Marcelleina tuberculispora	Denmark	AF335120
Mattirolomyces austroafricanus	South Africa	GQ231753
Mattirolomyces mexicanus	Mexico	HQ660379
Mattirolomyces mulpu	Australia	GQ231740
Mattirolomyces spinosus	USA	HQ660382
Mattirolomyces spinosus	Pakistan	HQ660385
Mattirolomyces spinosus	Pakistan	MT449183
Mattirolomyces terfezioides	Hungary	AY649792
Mattirolomyces terfezioides	France	GQ231755
Mycoclelandia arenacea	Australia	GQ231746
Peziza arvernensis	Denmark	AF335130
Peziza badiofusca	Sweden	AF335132
Peziza domiciliana	Denmark	AF335137
Peziza ellipsospora	USA	AF335139
Peziza emileia	Netherlands	AF335140
Peziza exogelatinosa	Denmark	AF335141
Peziza fimeti	Italy	KU898063
Peziza gerardii	Denmark	AF335142
Peziza gerardii	Denmark	AF335144
Peziza gerardii	Denmark	AY500546
Peziza lohjaoensis	Finland	KU898052
Peziza lobulata	USA	AY500548
Peziza nordica	Norway	KU898047
Peziza nivalis	USA	AF335154
Peziza oliviae	USA	KU898050

Table 1 Continued.

Species name	Geographic Origin	GenBank Accession number
Peziza ostracoderma	Netherlands	MH870829
Peziza phyllogena	USA	AY789328
Peziza proteana	Canada	AF335158
Peziza retrocurvata	Denmark	AF335159
Peziza subcitrina	Denmark	AF335162
Peziza subviolacea	Denmark	AF335165
Peziza varia	Spain	FN868867
Peziza vesiculosa	Denmark	AY500552
Plicaria carbonaria	USA	AY500533
Plicaria endocarpoides	USA	MN653021
Plicaria trachycarpa	Denmark	AY500554
Plicaria trachycarpa	Japan	AB828284
Ruhlandiella berolinensis	Canary Islands	AF335132
Ruhlandiella iophozoniae	Argentina	MG947625
Ruhlandiella patagonica	Chile	MG947609
Ruhlandiella patagonica	Chile	MG947612
Ruhlandiella truncate		MG947632
Sphaerosoma trispora	Australia	MH722262
Stouffera longii	USA	HQ660386
Temperantia tiffanyae	USA	HQ660387
Temperantia tiffanyae	USA	HQ660388
Terfezia aff olbiensis	Spain	HQ698062
Terfezia aff olbiensis	Spain	HQ698063
Terfezia alsheikhii	Spain	NG042571
Terfezia boudieri	Algeria	MF940208
Terfezia claveryi	Kuwait	AY500558
Terfezia crassiverrucosa	Algeria	MF940233
Terfezia leptoderma	Spain	HQ698057

Discussion

LSU sequence analyses of the new taxon, *Ahmadea dalanensis*, placed it within Pezizaceae in a strongly supported clade (99% bootstrap support, Fig. 1) that includes the truffle genera *Temperantia*, *Stouffera*, *Hydnobolites* (Kimbrough et al. 1991, Li et al. 2019), and *Delastria* (Alvarado et al. 2011). Their respective type species were identified as *T. tiffanyae*, *S. longii*, *H. cerebriformis* Tul. & C. Tul. and *D. rosea* Tul. & C. Tul. The topology of this tree is largely in agreement with that described in Kovács et al. (2011). Our choice of this LSU region for phylogenetic analyses follows the study on Pezizaceae by Hansen et al. (2001) which influenced many other studies on closely related genera, resulting in an extensive nrDNA LSU database for the Pezizaceae. Another study on Pezizaceae by Hansen et al. (2005) involved three regions: RPB2, LSU and β-tubulin. Although these authors found that RPB2 generally provide higher statistical support for clades, LSU was reported to be easier to amplify and nearly as useful. Moreover, Laessoe & Hansen (2007) analyzed LSU sequences of almost 200 species in their study on former Tuberales (now Pezizales).

The monospecific genera *Ahmadea* and *Temperantia* share some similar features such as having solid white gleba with fertile pockets that turn cream colored when dried, while the sterile veins remain unchanged. The absence of paraphyses and hyaline spores also make both genera similar. These two genera differ in their spore numbers and spore ornamentation. The two taxa *A. dalanensis* and *T. tiffanyae* (which was originally described as *M. tiffanyae* then renamed based on phylogenetic analyses; Kovács et al. 2011) differ in their asci, which are ellipsoid in *T. tiffanyae* whereas they are globose to subglobose in *A. dalanensis*. Additionally, the former revealed a lower spore number per ascus [1-3 (-4)], whereas the latter showed mostly 8 spored asci. *Ahmadea* shares

similar features with the monospecific genus *Stouffera* including a solid gleba, which is white when young and turns pale yellow at maturity. *Ahmadea* differs from *Stouffera* in its unusual double-spored ornamentation with small rounded hemispheres on the spore surface and between the walls of the reticulum. The description of *Delastria* Tul. & C. Tul indicates various shapes of asci from ovoid, oblong and curved to reniform containing 3 (-4) spino reticulated spores, whereas *Ahmadea* has mostly subglobose to globose asci and non-reticulate spores. The type species *Delastria rosea* has rose colored gleba but *A. dalanesis* has whitish gleba. With respect to some anatomical traits such as asci shape and spore shape and number, *Ahmadea* resemble *Hydnobolites* but there are prominent differences in the SEM images of the spore ornamentation, which is usually reticulate in *Hydnobolites*. Also *Hydnobolites cerebriformes* has lobed or folded ascomata which is different from those of *A. dalanensis* that have solid gleba with sterile veins and fertile portions.

The tree depicted in Fig. 1 indicates strong support for the monophyly between our samples and the monotypic genera *Temperantia* and *Stouffera* (96% bootstrap support). However, phylogenetic relationships among these taxa remain unresolved. This, along with marked LSU sequence differences (as indicated by the long branches leading to these taxa in Fig. 1) and significant anatomical differences have compelled us to create a new genus for our collections, *Ahmadea*.

Another finding during the current research study was the detection of non-orthologous ITS sequences in our samples, that precluded their analyses in a phylogenetic context. These non-orthologous or paralogous sequences are probably rDNA pseudogenes, which are probably not functional due to numerous mutations in the otherwise highly conserved 5.8S gene. Non-orthologous ITS sequences have been reported in other fungi, for instance, O'Donnell & Cigelnik (1997) identified the gene phylogenies of *Fusarium* from nr DNA ITS, mt SSU, β-tubulin gene and nuclear 28 S rDNA. All other gene regions were concordant except for nr DNA ITS, which showed discordance in the gene tree due to non-orthologous ITS2 types: type I and type II. Likewise, three non-orthologous ITS types were found in *Trichaptum abietinum*, a polypore fungus (Ko & Jung 2002).

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