MYCOLOGIA
2019, VOL. 111, NO. 2, 225–234
https://doi.org/10.1080/00275514.2018.1542864

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The *Cedrus*-associated truffle *Trappeindia himalayensis* is a morphologically unique and phylogenetically divergent species of *Rhizopogon*

Alija B. Mujic [a], Nan Zheng[a], Kristy Kim [a], Joseph W. Spatafora[b], Michael A. Castellano[c], and Matthew E. Smith [a]

[a]Department of Plant Pathology, University of Florida, Gainesville, Florida 32611; [b]Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331; [c]US Department of Agriculture, Forest Service, Northern Research Station, 3200 SW Jefferson Way, Corvallis, Oregon 97331

ABSTRACT

In the northwestern Himalayan mountains of India, the hypogeous sequestrate fungus *Trappeindia himalayensis* is harvested from forests dominated by the ectomycorrhizal tree *Cedrus deodara* (Himalayan cedar). This truffle has basidiospores that are ornamented with raised reticulation. The original description of *Trappeindia himalayensis* suggested that the gleba of this species is similar to young specimens of *Scleroderma* (Boletales), whereas its basidiospores are ornamented with raised reticulation, suggesting a morphological affinity to *Leucogaster* (Russulales) or *Strobilomyces* (Boletales). Given this systematic ambiguity, we have generated DNA sequence data from type material and other herbarium specimens and present the first molecular phylogenetic analysis of this unusual *Cedrus*-associated truffle. Despite the irregular ornamented basidiospore morphology, *T. himalayensis* is resolved within the genus *Rhizopogon* (Suillineae, Boletales) and represents a unique lineage that has not been previously detected. All known *Rhizopogon* species possess an ectomycorrhizal trophic mode, and because of its placement in this lineage, it is likely that *Trappeindia himalayensis* is an ectomycorrhizal partner of *Cedrus deodara*. This study highlights the importance of generating sequence data from herbarium specimens in order to identify fungal biodiversity and clarify the systematic relationships of poorly documented fungi.

ARTICLE HISTORY
Received 29 January 2018
Accepted 29 October 2018

KEYWORDS
Boletales; cryptic diversity; ectomycorrhiza; Himalayan cedar; sequestrate fungi; Suillineae; 1 new taxon

INTRODUCTION

Recent studies that have produced DNA sequence barcodes from fungal herbarium specimens and culture collections have discovered extensive "hidden" taxonomic and phylogenetic diversity (Brock et al. 2009; Nagy et al. 2011; Osmundson et al. 2013). For example, Brock et al. (2009) estimated that up to 70% of the taxonomic diversity in herbaria is not yet represented in GenBank. Based on nuc rDNA internal transcribed spacer (ITS1-5.8S-ITS2 = ITS) barcoding at the Kew Herbarium, they suggested that many of the "unknown" sequences in GenBank probably correspond to described fungal species or at least to specimens that had been previously collected and deposited in fungal herbaria. In a similar effort, Osmundson et al. (2013) generated some 1100 ITS sequences from the fungal collection at the Museum of Natural History in Venice. Their efforts resulted in sequences from 416 previously unrepresented taxa and 398 sequences whose best BLAST match was an unidentified environmental sequence. These findings corroborate the idea that many fungi are awaiting discovery in our herbarium cabinets.

Hypogeous sequestrate fungi (e.g., truffles) are excellent targets for sampling of molecular phylogenetic diversity from herbarium collections. Truffles have evolved independently within many different fungal lineages and are underrepresented in public DNA databases compared with epigeous fungal taxa (Trappe et al. 2009; Tedersoo et al. 2010). Because they reproduce belowground, truffles do not release sexually produced spores in the atmosphere, but these spores are often capable of surviving mammalian gut passage (Pyare and Longland 2001; Colgan and Claridge 2002; Izzo et al. 2005), persisting for long periods in the environment (Bruns et al. 2009; Nguyen et al. 2012), or surviving major disturbances such as wildfire (Glassman et al. 2016). All of these traits of truffles seem to be correlated with better long-term preservation of DNA in spores. However, because truffle tissues are often compressed, distorted, and morphologically quite different

CONTACT Alija B. Mujic amujic@csufresno.edu
Supplemental data for this article can be accessed on the publisher's Web site.

Published online 12 Feb 2019

from their closest epigeous relatives, it is challenging to determine their phylogenetic affinities based solely on morphology. In the past, many rare or unusual taxa were placed into large polyphyletic genera that actually contain species from multiple orders of fungi (Bougher and Castellano 1993; Smith et al. 2013). For example, several species that were once placed in the genus *Hymenogaster* (Agaricales) have now been placed, based on molecular phylogenetic analysis, in the Boletales (e.g., *Mycoamaranthus*), Gomphales (e.g., *Gautieria*), and Hysterangiales (e.g., *Aroramyces*), as well as to other lineages of Agaricales (e.g., *Descomyces* and *Descolea*) (Tedersoo et al. 2010; Tedersoo and Smith 2013).

Generating molecular data from truffle specimens stored in herbaria not only facilitates accurate identification of environmental samples but also resolves taxonomic issues and can clarify the trophic mode of a particular fungus. In a review of ectomycorrhizal (ECM) fungal diversity, Tedersoo et al. (2010) identified 33 fungal genera that were likely to be ECM but lacked sufficient sequence data; 21 of these genera included truffles and truffle-like fungi with unresolved phylogenetic relationships and unknown trophic modes. Although the majority of truffle species form ectomycorrhizas, it can be challenging to differentiate saprotrophic from biotrophic taxa, including those that are ECM. Recent studies have resolved the phylogenetic affiliations and trophic modes for both sequestrate ECM (e.g., *Fevansia* in the /albatrellus ECM lineage; Smith et al. 2013) and saprotrohic (e.g., *Amogaster* in the Agaricaceae and *Sedecula* in the Coniophoraceae; Ge and Smith 2013; Trappe et al. 2015) fungi from herbarium specimens.

Here, we address the phylogenetic relationships of the unusual truffle fungus *Trappeindia himalayensis*. This is the only species in the genus *Trappeindia* and is known only from low elevations at the base of the Himalayan mountains in India (Castellano et al. 2012). This truffle is collected in forests dominated by *Cedrus deodara* (Himalayan cedar, Pinaceae), an ECM conifer, and is consumed by locals, who refer to it as "Bankae" or "Janda." It possesses subglobose to globose basidiospores with reticulate ornamentation that led Castellano et al. (2012) to suggest a possible taxonomic affiliation with *Scleroderma* (Sclerodermataceae, Boletales), *Leucogaster* (Russulaceae, Russulales), or *Strobilomyces* (Boletaceae, Boletales) based upon the similarity of basidiospore morphology to taxa in these genera. However, our phylogenetic analyses indicate that this truffle species is nested within the genus *Rhizopogon* (Suillineae, Boletales) and represents a unique lineage that may be specifically associated with *Cedrus*. We suggest that *T. himalayensis* is an ECM associate of *Cedrus deodara* because members of the genus *Rhizopogon* are obligate ECM symbionts of Pinaceae host trees (Molina et al. 1999) and *Trappeindia himalayensis* has only been collected thus far in forests dominated by *Cedrus deodara* (Pinaceae).

MATERIALS AND METHODS

Specimens.—Three dried herbarium specimens of *T. himalayensis* (including the holotype and a paratype) were obtained from Oregon State University (OSC). Hand sections of specimens were mounted in water, Melzer's reagent, and 3% KOH and examined microscopically for morphological identification following Castellano et al. (2012). All other specimens examined were identified at least to genus level using the methods of Castellano et al. (1989) and other taxonomic resources (Smith and Zeller 1966; Arora 1986; Castellano et al. 1989; Trappe et al. 2009). Herbarium abbreviations follow Thiers [continuously updated]. The taxonomic description is taken and modified from Castellano et al. (2012).

Molecular protocols.—Fungal tissues were ground with liquid nitrogen in 1.5-mL Eppendorf tubes and DNA extracted with a cetyltrimethylammonium bromide (CTAB) phenol:chloroform:isoamyl alcohol protocol (Mujic et al. 2017). Polymerase chain reaction (PCR) amplification was attempted for eight loci using Phusion Hot Start II polymerase (ThermoFisher Scientific, Waltham, Massachusetts) with setup and reaction protocols recommended by the manufacturer. We amplified the following eight DNA regions using previously published primers (TABLE 1): mt encoded ATP synthase 6 (*ATP6*), the genes for RNA polymerase II largest (*RPB1*) and second largest (*RPB2*) subunits, translation elongation factor 1-alpha (*TEF1*), mt 23S rDNA (23S), nuc 18S rDNA (18S), nuclear 28S rDNA (28S), and ITS.

PCR amplicons were stained with SYBR Green I (Molecular Probes, Eugene, Oregon) and examined by electrophoresis on 1.5% agarose gels. Amplicons producing single discrete gel bands were cleaned with EXO (exonuclease I) and AP (Antarctic phosphatase) enzymes (Werle et al. 1994) and sequenced at the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR; http://www.biotech.ufl.edu/). ITS sequences of *T. himalayensis* specimens were compared with those in the National Center for Biotechnology Information (NCBI) GenBank database using BLASTn searches. These searches revealed high

Table 1. PCR Primer pairs used to amplify the selected loci.

Locus	Forward primer	Reverse primer	Forward primer reference	Reverse primer reference
ATP6	ATP6-1	ATP6-2	Kretzer and Bruns 1999	Kretzer and Bruns 1999
RPB1	RPB1-Af	RPB1-Cr	Matheny et al. 2002	Matheny et al. 2002
RPB2	fRPB2-5F, bRPB2-6F	bRPB2-7R	Liu et al. 1999; Matheny 2005	Matheny 2005
TEF1	EF1-1577F, EF1-986F	EF1-1567R, EF1-2218R	Rehner and Buckley 2005	Rehner and Buckley 2005
mtLSU	ML5	ML6	White et al. 1990	White et al. 1990
18S	NS1, NS5	SSU-1196, NS8	White et al. 1990	Borneman and Hartin 2000; White et al. 1990
28S	LROR	LR5	Bunyard et al. 1994	Vilgalys and Hester 1990
ITS	ITS1F	ITS4	Gardes and Bruns 1993	White et al. 1990

sequence similarity between *Trappeindia* and species of *Suillus* and *Rhizopogon* (Suillineae). Accordingly, DNA was extracted from additional *Suillus* and *Rhizopogon* herbarium specimens, and PCR amplification was attempted for all eight loci (TABLE 2).

Phylogenetic analyses.—Sequence data of the loci above from *Rhizopogon* and *Suillus*, as well as Boletales outgroup taxa, were derived from the data sets of Binder and Hibbett (2006) and Skrede et al. (2011). In addition to Sanger sequences from herbarium specimens, we mined sequence data from all five subgenera of *Rhizopogon* (Grubisha et al. 2002) from the genomes of ten *Rhizopogon* taxa using the methods and data set of Mujic et al. (2019). Additional *Rhizopogon* sequence data were mined from the genome sequence of *R. vesiculosus* (Mujic et al. 2017). Nucleotide sequence data were also mined from the genomes of *Suillus luteus* (Kohler et al. 2015) and *S. brevipes* (Branco et al. 2015) available through the US Department of Energy Joint Genome Institute (JGI) MycoCosm portal (http://genome.jgi.doe.gov/programs/fungi/index.jsf) (SUPPLEMENTARY TABLE 1).

Sequence data for each locus were individually aligned using MUSCLE (Edgar 2004) and manually adjusted in the software package MESQUITE (Maddison and Maddison 2010). The most appropriate model of evolution was chosen for each individual locus using jModelTest (Posada 2008). Maximum likelihood (ML) phylogenetic analyses were performed for each locus in RAxML 7.04 (Stamatakis 2008) using the GTRGAMMA model of evolution and 1000 bootstrap replicates. Resulting trees from these analyses did not produce differing well-supported topologies (>70% bootstrap support). Accordingly, individual locus alignments were combined into a single concatenated supermatrix alignment, with missing data for individual taxa treated as gaps. The supermatrix was then analyzed using MrBayes 3.2 (Ronquist et al. 2012) as implemented on the CIPRES science portal (Miller et al. 2010) and RAxML. Bayesian inference (BI) analysis utilized two independent runs of four Markov chains that each ran for 1 million generations with tree sampling every 1000 generations and a burn-in of 25% of the initial sampled trees. The supermatrix was partitioned by locus for BI analysis, and each partition was assigned the best-fit model according to jModelTest. ML analysis of the unpartitioned supermatrix was run in RAxML with the same parameters that were used for individual alignments. The supermatrix alignment is available on TreeBASE (https://treebase.org) under accession number S22235.

RESULTS

Phylogenetic analyses.—We performed BLASTn searches of our *Trappeindia himalayensis* ITS sequences against the GenBank database and found that *Suillus* and *Rhizopogon* were the only high-scoring hits recovered. The top three BLASTn hits to the ITS sequence from the *Trappeindia himalayensis* holotype specimen (HPUB 2645, Singh 2645) were KT968584 *Rhizopogon atroviolaceus* ITS (e-value = 0.0, identity = 95%), KT968574 *Rhizopogon atroviolaceus* ITS (e-value = 0.0 identity = 95%), and KC346844 *Rhizopogon atroviolaceus* ITS (e-value = 0.0, identity = 95%). We produced sequence data from six *Rhizopogon*, four *Suillus*, and three *T. himalayensis* herbarium specimens (81 new sequences in total) and mined sequence data from 11 *Rhizopogon* genomes and two *Suillus* genomes (TABLE 2, SUPPLEMENTARY TABLE 1). Newly generated sequence data were aligned into an eight-locus supermatrix with sequence data from 13 specimens previously analyzed by Skrede et al. (2011) and Binder and Hibbett (2006) (TABLE 2). Both ML and BI analyses generated a well-resolved phylogeny with support for the placement of *T. himalayensis* within the genus *Rhizopogon* (FIG. 1). *Trappeindia himalayensis* is a distinct lineage that was resolved as sister to subgenera *Rhizopogon* and *Versicolores* with strong statistical support.

TAXONOMY

Rhizopogon himalayensis (Castellano, S.L. Miller, Singh & Lakhanpal) A.B. Mujic & M.E. Sm., comb. nov. FIG. 2B, C, E

Table 2. GenBank accession numbers for nucleotide sequence data generated in this study from herbarium specimens (in bold) and nucleotide sequence data derived from external studies.

Sequence data generated in this study from herbarium specimens

Genus	Species	*Rhizopogon* subgenus	Herbarium[a]	Collector's number	ITS	SSU	LSU	*TEF1*	*RPB1*	*ATP6*	mtLSU	*RPB2*
Rhizopogon	*cf roseolus*	*Roseoli*	FLAS	MES1399	**MH878761**	**MH878793**	**MH878777**	**MH890602**	**MH890593**	NA	NA	**MH890583**
Rhizopogon	sp.	*Amylopogon*	FLAS	MES1400	**MH878762**	**MH878794**	**MH878778**	**MH890603**	**MH890594**	**MH890572**	**MH880262**	**MH890584**
Rhizopogon	sp.	*Roseoli*	FLAS	MES429	**MH878757**	**MH878786**	**MH878772**	**MH890598**	**MH890589**	NA	NA	**MH890579**
Rhizopogon	sp.	*Rhizopogon*	FLAS	MES795	**MH878758**	**MH878787**	**MH878773**	**MH890599**	**MH890590**	NA	**MH880263**	**MH890580**
Rhizopogon	sp.	*Rhizopogon*	FLAS	MES818	**MH878760**	**MH878789**	**MH878775**	**MH890601**	**MH890592**	NA	**MH880265**	**MH890582**
Rhizopogon	*subaustralis*	*Versicolores*	FLAS	MES798	**MH878759**	**MH878788**	**MH878774**	**MH890600**	**MH890591**	**MH890574**	**MH880264**	**MH890581**
Suillus	*lakei*	NA	FLAS	AR15-015	**MH878754**	**MH878790**	**MH878769**	**MH890596**	**MH890586**	NA	**MH880260**	**MH890577**
Suillus	*lakei*	NA	FLAS	AR15-018	**MH878755**	**MH878791**	**MH878770**	NA	**MH890587**	NA	**MH880261**	**MH890578**
Suillus	*luteus*	NA	FLAS	AR15-026	**MH878756**	**MH878792**	**MH878771**	NA	**MH890588**	NA	NA	NA
Suillus	*salmonicolor*	NA	FLAS	MES1445	**MH878763**	**MH878795**	**MH878776**	**MH890597**	**MH890595**	**MH890573**	NA	**MH890585**
Trappeindia	*himalayensis*	NA	OSC	Singh 2645 (**Holotype**)	**MH878753**	**MH878785**	**MH878767**	**MH890604**	NA	NA	**MH880258**	**MH890575**
Trappeindia	*himalayensis*	NA	OSC	TNL1988	**MH878764**	**MH878783**	**MH878768**	**MH890605**	NA	NA	**MH880259**	**MH890576**
Trappeindia	*himalayensis*	NA	OSC	Trappe31890, Singh 2644 (**Paratype**)	**MH878765**	**MH878784**	**MH878766**	NA	NA	NA	NA	NA

Sequence data derived from external studies

Genus	Species	Isolate ID	Specimen number	Reference[b]	ITS	SSU	LSU	*TEF1*	*RPB1*	*ATP6*	mtLSU	*RPB2*
Rhizopogon	*olivaceotinctus*	NA	OSC8245	Binder and Hibbett (2006)	See reference	DQ534690	See reference	NA	NA	See reference	See reference	NA
Suillus	*pictus*	NA	AFTOL-717	Skrede et al. (2011)	AY854069	AY662659	AY684154	AY883429	NA	NA	NA	AY786066
Suillus	*ochraceoroseus*	SAR-84-137	NA	Binder and Hibbett (2006)	See reference	NA	See reference	NA	NA	See reference	See reference	NA
Suillus	*granulatus*	REG Sg1	NA	Binder and Hibbett (2006)	See reference	DQ534691	See reference	NA	NA	See reference	DQ534592	NA
Suillus	*lakei*	PDD7	NA	Binder and Hibbett (2006)	See reference	DQ534692	See reference	NA	NA	See reference	See reference	NA
Suillus	*variegatus*	REG Sv3	NA	Binder and Hibbett (2006)	See reference	DQ534693	See reference	NA	NA	See reference	DQ534593	NA
Leucogyrophana	*mollusca*	DAOM138006	NA	Binder and Hibbett (2006)	See reference	DQ534684	See reference	NA	NA	NA	DQ534590	NA
Hygrophoropsis	*aurantiaca*	MB 03-127	NA	Binder and Hibbett (2006)	AY854067	AY662663	AY684156	NA	NA	DQ534605	See reference	NA
Coniophora	*arida*	FP-104367	NA	Binder and Hibbett (2006)	See reference	See reference	See reference	NA	NA	See reference	See reference	NA
Coniophora	*marmorata*	DAOM178982	NA	Binder and Hibbett (2006)	See reference	See reference	See reference	NA	NA	NA	DQ534585	NA

[a]Herbarium codes: FLAS = Fungal Collection of the Florida Museum of Natural History; OSC = Oregon State University Fungal Herbarium.
[b]GenBank accession numbers are listed when the reference study generated the sequence data for their data set. If "see reference" is listed in place of an accession number, this locus data were generated by a study other than the reference study but is referenced in the reference study.

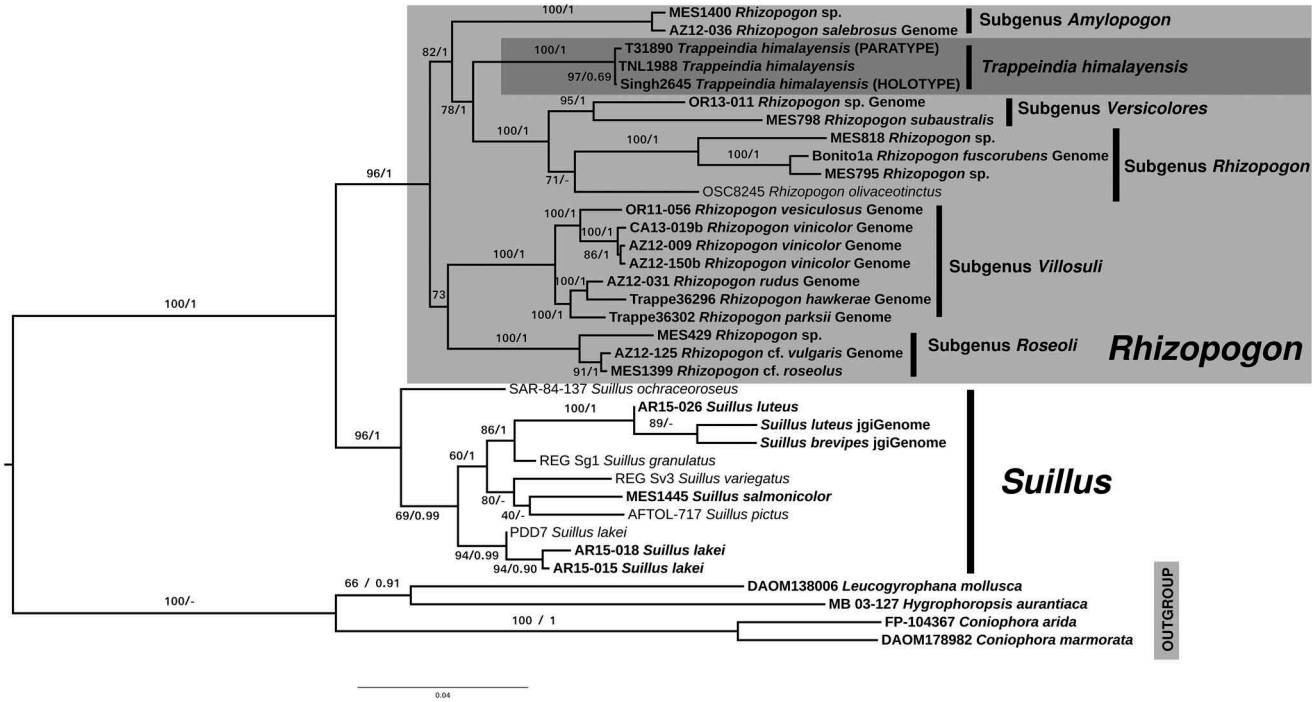

Figure 1. ML Phylogram of *Suillus* and *Rhizopogon* based on eight loci (*ATP6, TEF1, RPB1, RPB2*, mt 23S, and nuc 18S, 5.8S, and 28S rDNA). Taxa representing sequence data generated for this study are shown in boldface. Maximum likelihood (ML) bootstrap support values (left of slashes) and Bayesian inference (BI) posterior probabilities (right of slashes) are shown for all branches. *Hygrophoropsis* and *Leucogyrophana* (Hygrophoropsidaceae) and *Coniophora* (Coniophoraceae) were used as outgroup taxa. The scale bar indicates the expected number of substitutions per site.

MycoBank MB820437

≡ *Trappeindia himalayensis* Castellano, S.L. Miller, Singh & Lakhanpal, Kavaka 40:3. 2012. Basionym.

Basidiomata 0.5–4 cm, subglobose to globose or slightly lobed. Peridium dark brown to black, drying to a glabrous surface, with a distinct basal point of attachment. Gleba loculate, locules polygonal to elongate, empty with basidiospores lining surface, moist, tramal tissue white, basidiospores brown, as dried hard, brittle and pale to dark brown, columella absent. Rhizomoprh single, stout, ±1 mm diam, concolorous with peridium, attached at base of basidiomata. Taste of boiled potatoes. Odor not distinctive.

Peridium 140–300 µm thick, composed of thin-walled, frequently branched, coarse, and loosely interwoven hyphae, 3–5 µm diam, brown near surface, hyaline towards gleba, incorporating debris or with dark brown pigment crystals. Trama hyaline, gelatinized, forming a loosely interwoven mediostratum of highly branched, occasionally irregularly swollen, hyphae, 2.5–4.5 µm diam, divergent. Basidiospores 21–24(–28) µm diam (mean 23 µm), globose to occasionally subglobose, symmetrical; ornamentation an irregular reticulation, aveolae 2–5 µm diameter and 2.5–3.5 µm high. The walls of the aveolae up to 1.5 µm broad, often curved at apex. Sterigmal attachment occasionally evident and up to 5 µm broad. Basidiospores hyaline to pale brown in KOH when young, brown when mature; inamyloid and nondextrinoid, yellow-brown in Melzer's reagent. Basidia 40–45 × 5–8 µm, 1- or 2-spored, in a palisade, cylindrical to irregularly elongate, thin-walled, hyaline; sterigmata up to 6.5 µm broad and up to 24 µm long, collapsing when spores detach. Basidioles 10–15 × 5–8 µm, hyaline, clavate. Cystidia absent. Clamp connections absent.

Ecology and distribution: Hypogeous under *Cedrus deodora*, northern India, Apr.

Specimens examined: INDIA. HIMACHAL PRADESH: Junee Valley, Mandi Devidar, hypogeous under *Cedrus deodara*, 16 Apr 1989 (**holotype** HPUB 2645, **isotype** OSC); same locality as above, 15 Apr 1989 (HPUB 2644, Trappe 31890 OSC); near Shimla, 1988 (TNL 1988, OSC).

DISCUSSION

We have generated the first molecular phylogeny of *Trappeindia* and determined that the only species in the genus represents a unique lineage of *Rhizopogon*. Consequently, the species is transferred to *Rhizopogon*, and *Trappeindia* becomes a synonym of *Rhizopogon*. This determination is made using sequence data and morphological examination of the holotype specimen,

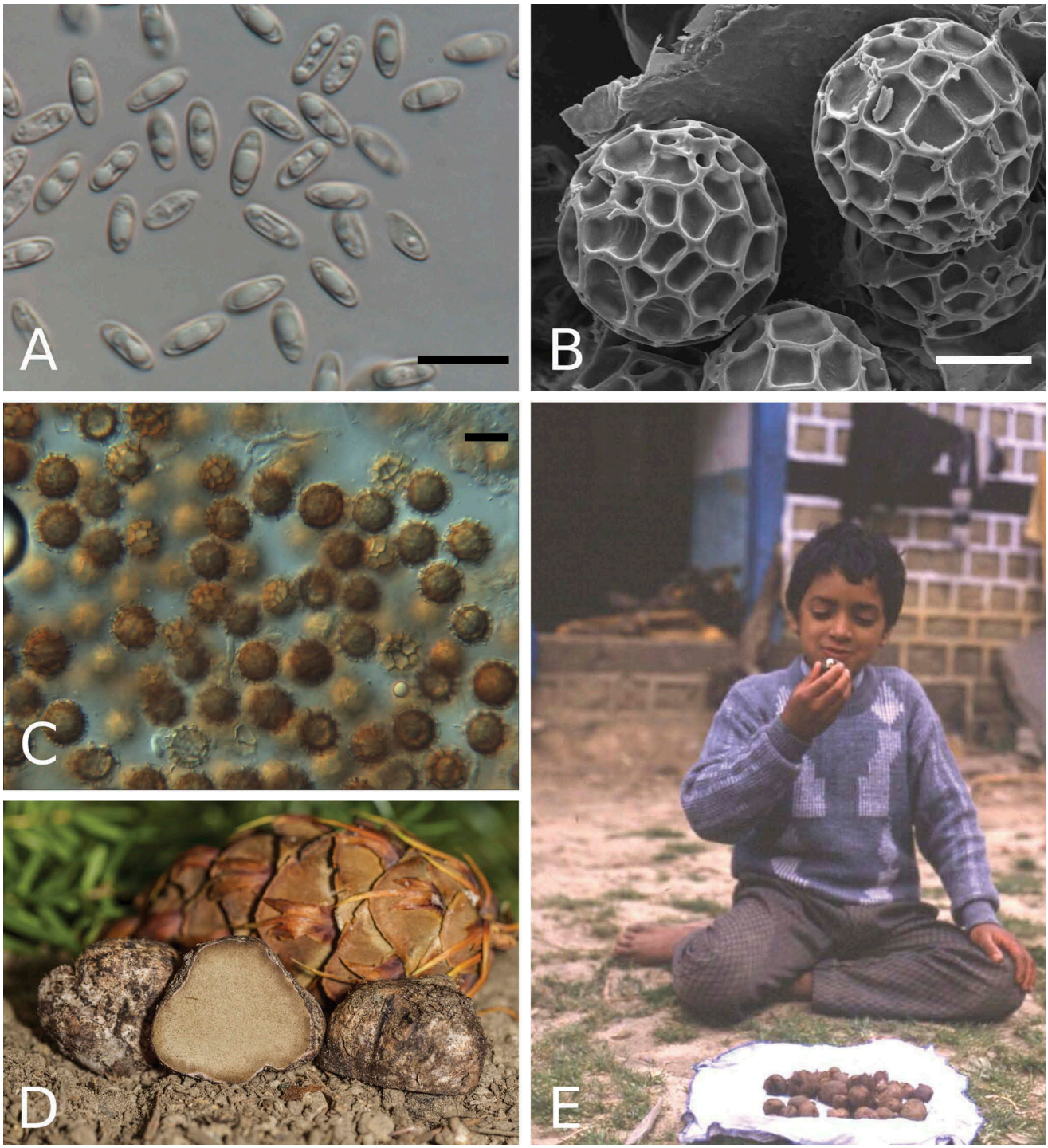

Figure 2. *Rhizopogon himalayensis* basidiospores and basidiomata contrasted with those of other *Rhizopogon* species. A. Typical basidiospores of *Rhizopogon* (*R. togasawariana*). B. Scanning electron microscopic image of *Rhizopogon himalayensis* basidiospores (Trappe 31890, paratype). C. Light microscopic image of *Rhizopogon himalayensis* basidiospores (Trappe 31890, paratype). D. Basidiomata of *R. villosulus*. E. Boy admiring basidiomata of *R. himalayensis*. Bars: A, B = 10 µm; C = 20 µm.

a paratype specimen, and a third previously unpublished specimen of *Trappeindia himalayensis*. Although the phylogenetic placement of *Trappeindia* within *Rhizopogon* is a clear conclusion of both ML and BI phylogenetic analyses (FIG. 1), there are several morphological and ecological traits of *Trappeindia* that are otherwise unique within *Rhizopogon*. Primary among these traits is the morphological divergence of *Trappeindia* basidiospores from those of *Rhizopogon*.

Rhizopogon himalayensis possesses a divergent basidiospore morphology from all other known *Rhizopogon* species. All previously described species of *Rhizopogon*

possess smooth ellipsoid basidiospores or smooth ellipsoid-truncate basidiospores bearing two prongs along the edge of the basal truncation (Smith and Zeller 1966; Smith et al. 1981). *Rhizopogon himalayensis* basidiospores are unique in this regard because they are globose to subglobose and ornamented by fused ridges that form a complete reticulum. The morphology and inamyloid reaction of *R. himalayensis* basidiospores are similar to those of some *Stobilomyces* species (Boletales, Boletaceae), which led Castellano et al. (2012) to suggest a taxonomic affinity between *Trappeindia* and *Strobilomyces*. However, our molecular phylogenetic analyses do not support this hypothesis. We conclude that the reticulate inamyloid basidiospores of *R. himalayensis* represent an independent evolutionary derivation and convergent morphology with *Strobilomyces.*

The evolution of unique spore ornamentation in a group of fungi where all closely related taxa have smooth spores is not unusual. One example of this evolutionary pattern is *Guyanagaster* (Henkel et al. 2010). This genus contains two species of truffles with highly ornamented spores, but all other known relatives are agaricoid taxa with smooth spores (Koch et al. 2017). Another example is the truffle genus *Heliogaster*. The only known species, *H. columellifera*, has ornamented spores that are similar to those of *Octaviania* species. However, *Heliogaster* is closely related to *Xerocomellus* as well as members of several other agaricoid bolete genera that produce smooth basidiospores (Orihara et al. 2010). A third example is the sequestrate genus *Lycoperdon*, which is a member of the Agaricaceae (Vellinga 2004; Vellinga et al. 2011). Most species of Agaricaceae form typical epigeous mushrooms with smooth basidiospores, but *Lycoperdon* produces gasteroid fruiting bodies with ornamented spores.

In the case of sequestrate fungi, there may be an even greater chance for the divergence of basidiospore morphology between closely related taxa due to the loss of selective pressures associated with the maintenance of ballistospory (forcible spore discharge). Epigeous Agaricomycetes are likely under strong selection to retain basidiospore morphologies optimal for aerial spore dispersal (Money 1998; Pringle et al. 2005; Fischer et al. 2010). Because truffles and other sequestrate fungi are not under selective pressure to produce sexual spores actively discharged from basidia, they might be more likely to evolve new and divergent spore morphologies adapted to passive spore dispersal mechanisms such as animal mycophagy and/or dormancy in spore banks (Fogel and Trappe 1978). *Rhizopogon* species are known to rely upon passive spore dispersal mechanisms, but the only species known to have ornamented spores is *R. himalayensis*. It is unclear why other members of genus *Rhizopogon* would lack basidiospore ornamentation if this trait evolved under selective pressure for passive spore dispersal. Thus, it is likely that spore ornamentation in *R. himalayensis* evolved under selective pressures that are different from those of other *Rhizopogon* species. However, it should be noted that although external ornamentation of *Rhizopogon* basidiospores has not previously been observed, scanning electron micrographs of *Rhizopogon* basidiospores with the outer perisporium layer removed revealed subsurface ornamentation (Martin 1996). This indicates that the ornamentation of *R. himalayensis* basidiospores may not be unique within the genus *Rhizopogon*. It is possible that *R. himalayensis* may have lost the perisporium layer rather than the independent evolution of external basidiospore ornamentation. Additional electron microscopy studies of *Rhizopogon* basidiospores are needed to test this hypothesis.

Because all known species of *Rhizopogon* are ECM and because *R. himalayensis* is only known from forests where *C. deodara* is the dominant ECM host tree, we can only conclude that *R. himalayensis* is an ECM symbiont of *C. deodara*. If true, this host association would be unique within *Rhizopogon*, which is otherwise restricted primarily to host trees in the genera *Abies, Picea, Pinus, Pseudotsuga*, and *Tsuga* (Molina and Trappe 1994; Molina et al. 1999). Although the putative host association of *Rhizopogon* with *Cedrus* is novel, it is not surprising. The genus *Cedrus* is a member of family Pinaceae, which also contains the principle host trees of *Rhizopogon* species (Lin et al. 2010). This finding is also consistent with the host preferences of other Suillineae fungi, most of which associate with Pinaceae. We suggest that *R. himalayensis* displays host specificity for *Cedrus* much like *Rhizopogon* subgenus *Villosuli* does for *Pseudotsuga* (Molina and Trappe 1994; Grubisha et al. 2002; Mujic et al. 2019). Phylogenetic placement within *Rhizopogon* is strong evidence that *R. himalayensis* possesses a biotrophic ECM habit, but other methods could be used to demonstrate this ecology and the host affiliation with *Cedrus* such as ECM pure synthesis studies (Molina and Trappe 1994), direct observation of mycelial connectivity between basidiocarps and ECM root tips, DNA sequencing directly from colonized ECM roots, and/or stable isotope analyses of carbon and nitrogen.

It would seem that *R. himalayensis* warrants systematic placement in a new subgenus of *Rhizopogon* based on the unique basidispore morphology, but we reserve this task for a date when more complete

sampling of the genus can be performed. Although extensive surveys of epigeous ECM fungi have been conducted in natural and managed stands of *Cedrus atlantica* in north Africa (Nezzar-Hocine et al. 1996, 1998) and *C. deodara* in Asia (Hanif et al. 2012; Itoo and Reshi 2014), very little is known about the truffle taxa that form ECM relationships with *Cedrus* species. Given the high taxonomic diversity of *Rhizopogon*, the novelty of the putative *Cedrus* ECM association in *Rhizopogon*, and the degree of host specificity, it is likely that other undiscovered members of *Rhizopogon* occur in ECM association with *Cedrus*.

ACKNOWLEDGMENTS

We would like to thank the herbarium at Oregon State University (OSC) for lending specimens. We thank two anonymous reviewers and the editor for constructive comments that improved the quality of this work.

FUNDING

Funding was provided by a National Science Foundation grant (DEB-1354802) to M.E.S. and P. B. Matheny, with additional support from the University of Florida's Institute for Food and Agricultural Sciences (IFAS). Participation by K. Kim was made possible in part by the University of Florida Student Science Training Program (SSTP) to prepare advanced high school students for careers in science.

ORCID

Alija B. Mujic http://orcid.org/0000-0002-5810-5521
Kristy Kim http://orcid.org/0000-0002-3869-4939
Matthew E. Smith http://orcid.org/0000-0002-0878-0932

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