



GLOBAL JOURNAL OF SCIENCE FRONTIER RESEARCH: C
BIOLOGICAL SCIENCE

Volume 21 Issue 3 Version 1.0 Year 2021

Type: Double Blind Peer Reviewed International Research Journal

Publisher: Global Journals

Online ISSN: 2249-4626 & Print ISSN: 0975-5896

New Molecular Data in the Truffle-Like Fungus, *Aroramyces Herrerae*, Reveal A Novel Cryptic Ancestral Taxon, *Pterosporomyces Herrerae* Gen. Nov. & Comb. Nov. (Trappeacea, Phallales)

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Abstract- Recently, *Aroramyces herrerae* was described based on morphology, ecology, and taxonomically placed in the family Hysterangiaceae (Hysterangiales). However, a DNA analysis revealed that *A. herrerae* belongs to a new sequestrate cryptic genus, *Pterosporomyces*, in the family Trappeaceae (Phallales), nested with *Restingomyces reticulatus* and *Phalobata alba* and jointly forming a basal clade of Phallales with strong statistical support. *Pterosporomyces herrerae* gen. nov & comb. nov. is characterized by an olive green to brown gleba and wing spores (utriculum) up to 6 μm broad and its ITS and ATP6 variation. *Pterosporomyces* is similar in morphology to *Restingomyces* but differs by the spore ornamentation been utriculate in the former and alveolate in the latter.

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GJSFR-C Classification: FOR Code: 279999



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New Molecular Data in the Truffle-Like Fungus, *Aroramycetes Herrerae*, Reveal a Novel Cryptic Ancestral Taxon, *Pterosporomyces Herrerae* Gen. Nov. & Comb. Nov. (Trappeaceae, Phallales)

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Abstract- Recently, *Aroramycetes herreriae* was described based on morphology, ecology, and taxonomically placed in the family Hysterangiaceae (Hysterangiales). However, a DNA analysis revealed that *A. herreriae* belongs to a new sequestrate cryptic genus, *Pterosporomyces*, in the family Trappeaceae (Phallales), nested with *Restingomyces reticulatus* and *Phalobata alba* and jointly forming a basal clade of Phallales with strong statistical support. *Pterosporomyces herreriae* gen. nov. & comb. nov. is characterized by an olive green to brown gleba and wing spores (utriculum) up to 6 μm broad and its ITS and ATP6 variation. *Pterosporomyces* is similar in morphology to *Restingomyces* but differs by the spore ornamentation been utriculate in the former and alveolate in the latter. A taxonomic amendment is presented for the family Trappeaceae to include the new genus and recombine *Aroramycetes herreriae* into *Pterosporomyces herreriae* gen. nov. & comb. nov. and a redescription with new morphological, rDNA, and mtDNA data with illustrations are provided.

Keywords: fungi evolution, truffle, angiocarpic fungi, hypogeous fungi.

I. INTRODUCTION

Aroramycetes herreriae Guevara, Gómez & Castellano, and *A. balanosporus* Guevara & Castellano were described for the family Hysterangiaceae based mainly on morphology and ecology without DNA support (Guevara et al. 2016). However, a recent ATP6 and ITS genes analysis was

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performed on these two species to corroborate their novel status. The phylogenetic analysis showed that, in fact, *A. balanosporus* belongs to the family Hysterangiaceae as expected; however, in *A. herreriae*, the ITS and ATP6 analysis indicated that this taxon belongs to the family Trappeaceae into the order Phallales. Based on this, the recombination of *A. herreriae* to the new genus *Pterosporomyces herreriae* is proposed here. This new genus, among other hypogeous and stinkhorn fungi, belongs to the monophyletic subclass Phallomycetidae which is one of the most amazing fungi in recent mycological research due to the great diversity and evolutionary forms present in this group (Hosaka et al. 2006; Trappe et al. 2009). The family Trappeaceae, which includes *Trappea darkeri* (Zeller) Castellano, *Restingomyces reticulatus* Sulzbacher, B.T. Goto & Baseia, *Phallobata alba* G. Cunn., and the new taxon *Pterosporomyces herreriae* is ancestral in the Order Phallales (Hosaka et al. 2006; Sulzbacher et al. 2016). This research will increase our taxonomic and systematic understanding of the Gomphales and Phallales in North America.

II. MATERIAL AND METHODS

Basidiomata sampling, macro and microscopic analysis were performed following the recommendations of Castellano et al. (1989) and Pegler et al. (1993). The colors of fresh fruiting bodies are in general terms of the authors. For microscopic observations herborized specimens were hand-cut and mounted in 5% KOH, Melzer's reagent, or water. Dried and herborized specimens are deposited at José Castillo Tovar herbarium, ITCV. Thirty measurements including means were obtained from mature basidiospores with a compound microscope at 1000 × under oil immersion. DNA sequencing and phylogenetic analyses.—Molecular protocols follow those of Guevara et al. (2008). First, DNA was obtained from basidiomata by the chloroform extraction technique using CTAB buffer.

The internal transcribed spacer (ITS) region was amplified with the primer set ITS1f- ITS4 (White et al. 1990, Gardes and Bruns 1993). Then, the ATP6 gene was amplified with the primer set atp6-1 and atp6-2 (Kretzer and Bruns. 1999). Amplicons were cleaned enzymatically with antarctic phosphatase and endonuclease (New England Biolabs, Ipswich, Massachusetts). Posteriorly, Sanger sequencing was performed by Big Dye chemistry 3.1 (Applied Biosystems, Foster City, California) with the forward primer ITS or atp6-1 and reverse primers ITS4 or atp6-2. In addition, the DNA sequences were determined on an ABI 3700 capillary sequencer (Applied Biosystems, Foster City, California), viewed and manually edited in Sequencher 4.0 (Gene Codes Corp., Ann Arbor, Michigan). Later, sequences were queried against GenBank with the BLASTN algorithm to verify that sequences belonged to *Aroramyces*. MUSCLE (Edgar 2004) was used to align sequences and manually checked, and ambiguities were excluded in Mesquite 2.5 (Maddison and Maddison 2009).

Furthermore, the phylogenetic analyses were performed with maximum likelihood (ML) in PAUP* (Swofford 2002), and Bayesian inference (BI) with MrBayes (Huelsenbeck and Ronquist 2001). The Akaike criterion model (best-fit nucleotide substitution) information was selected and executed in PAUP* 4d106 (Swofford 2002). ML bootstrap support based on 1000 replicates was assessed with RAxML (Stamatakis et al. 2008). BI analyses and posterior probability were run through the CIPRES Web portal (<http://www.phylo.org/>). BI was based on parallel runs of 20 million generations sampling every 1000 generations for the phylogenetic tree.

Finally, sequences of this study were uploaded in GenBank under accession numbers MZ343611, MZ343612, and MK811032,

III. RESULTS

a) *Molecular analyses*

A total of 49 sequences published or reported in the NCBI were selected for the ITS and ATP6 phylogenetic analysis (Table 1 & 2). As previous studies have shown, the family Trappeaceae is a basal clade in the Phallales according to ML and Bayesian Inference analyses (Fig. 1 & 2). The Bayesian inference analysis recovered *Pterosporomyces herrerae* as a monophyletic group. The amendment to accommodate *Aroramyces herrerae* to *Pterosporomyces herrerae* in the basal family Trappeaceae is proposed as a new combination supported by ITS and ATP6 analysis with strong statistical support (PP=1), the internal nodes of *Pterosporomyces* were well supported too, in addition to new morphological characters.

IV. TAXONOMY

Trappeaceae P.M. Kirk *In*: Kirk PM, Cannon PF, Minter DW, Stalpers JA, eds. 2008. Dictionary of the Fungi. 10th ed.

a) *Taxonomic amendment of the family Trappeaceae to include Pterosporomyces*

Basidiomata hypogeous, sequestrate, gregarious, scattered, globose, subglobose or irregular, white pale tan to brownish, mottled dark brown with pale areas when handling and when dried, smooth to slightly tomentose, with mycelial strands, odor acetone/ether solvent-like. Peridium two layers (separable from the gleba when dried). Gleba green, brown or blackish, locules ellipsoid to elongate, columella dendroid, gelatinized, grayish, rhizomorphs few, attached at the base. Basidiospores small, smooth, reticulate or utriculate, (wing-like ornamentation), fusoid, ellipsoids, up to (5) 6 μ m broad laterally, numerous fine spines within the "utriculum," the utriculum encompassing hilar appendage to give a truncated appearance, utriculum often no evenly inflated to protrude from one side or another, occasionally surrounding entire spore originating from the spore base, pale brown in mass in KOH, inamyloid, nondextrinoid.

Type genus: Trappea Castellano

Other genera: Pterosporomyces Guevara, Gomez & Z.W. Ge, *Restingomyces* Sulzbacher, T. Grebenc & Baseia and *Phallobata* G. Cunn.

Pterosporomyces Guevara, Gómez, & Z.W. Ge gen. nov.

Mycobank: 834809 GenBank: MZ343611, MZ343612 & MK811032

Typification: Pterosporomyces herrerae (Guevara, Gómez, Castellano) Guevara, Gómez & Z.W. Ge

Etymology: "in reference to a fungus with wing (utricle) spore " the genus is dedicated to the pioneer mycologist from northern Mexico José Castillo Tovar.

Diagnosis: The genus differs from all other known Phallales by the spores with distinct inflated utricule laterally, and *ITS* and *ATP6* analysis placed this taxon into the family Trappeaceae.

Pterosporomyces herrerae (Guevara, Gómez, Castellano) Guevara, Gómez & Z.W. Ge, *gen. nov.* & *comb. nov.*

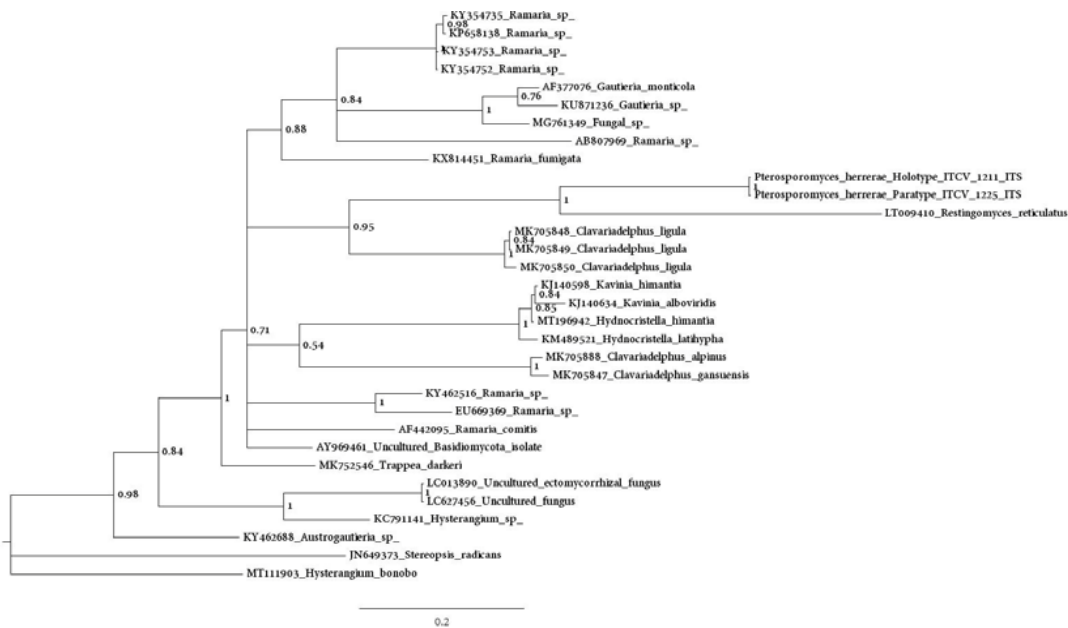


Figure 1: *Pterosporomyces herrerae*, phylogenetic tree inferred under the maximum-likelihood (ML) criterion for ITS gene with Mr. Bayes. The posterior probabilities for each clade are shown on the branches. The accession numbers in the sequence labels indicate the GenBank accession numbers

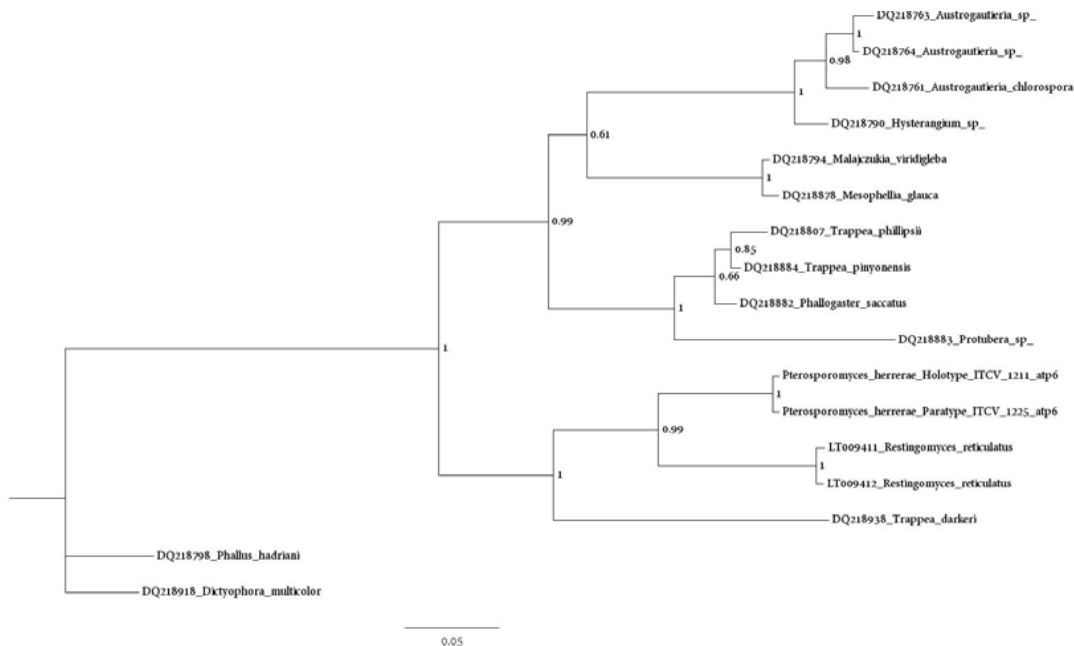


Figure 2: *Pterosporomyces herrerae*, phylogenetic tree inferred under the maximum-likelihood (ML) criterion for ATP6 gene with Mr. Bayes. The posterior probabilities for each clade are shown on the branches. The accession numbers in the sequence labels indicate the GenBank accession numbers

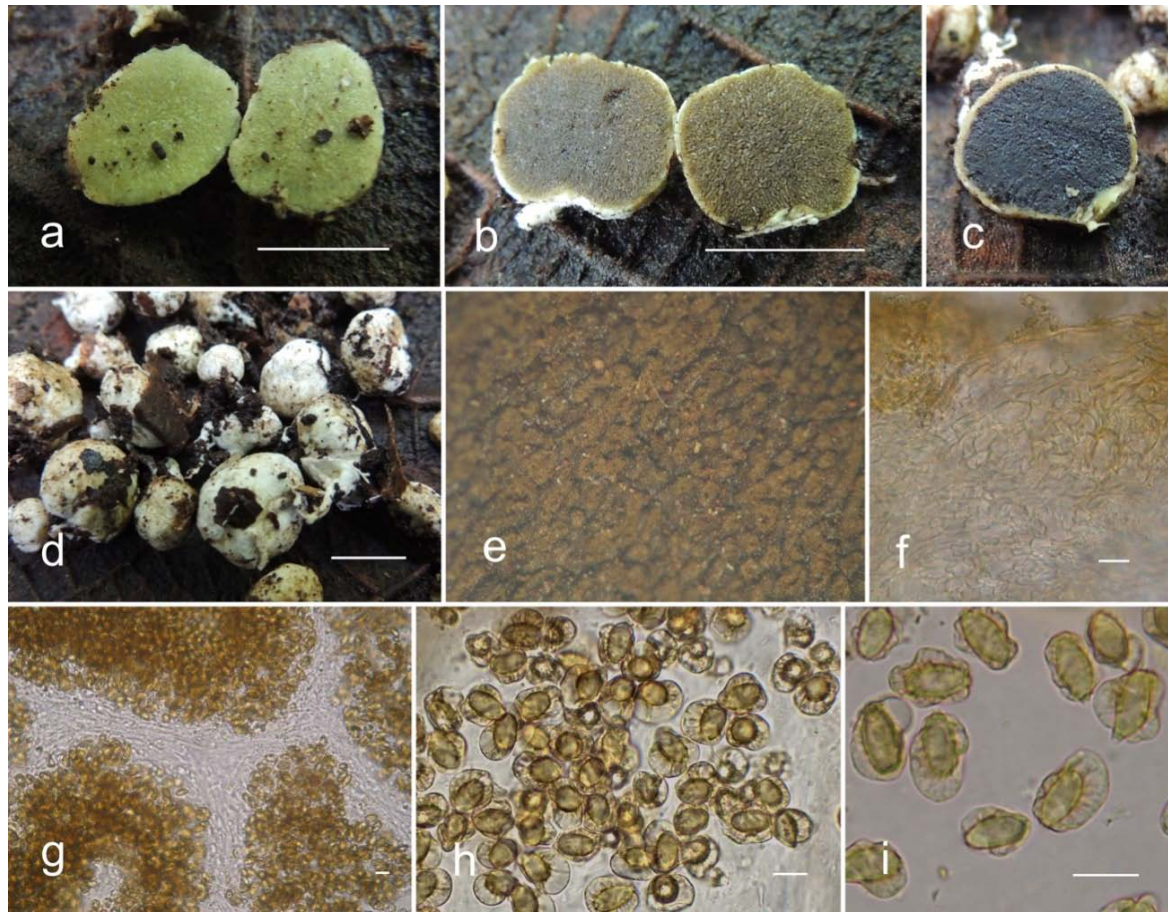


Figure 3: a-i, *Pterosporomyces herrerae* comb. nov. (ITCV 1211 Typus), a, b, & c: cross-sections of basidiome (bar = 1 cm), d: basidiomata (bar = 1 cm), e: close up of gleba, f: epicutis with pseudoparenchyma cells, (bar = 10 μ m), g: Trama (bar = 10 μ m), h & i: basidiospores with inflated utricles (bar 10 = μ m)

= *Aroramyces herrerae* Guevara, Gómez & Castellano, IMA Fungus 7(2) 235-238, 2016 (basionimo)

MB 812928, GenBank: MZ343611 (Type), MZ343612 (paratype) & MK811032

Holotype Guevara 1211 (ITCV1211), paratype Guevara 1225 (ITCV 1225)

Macromorphology. Fruiting bodies 5-18 x 6-15 x 5-10 mm, irregular, globose or subglobose. Basidiomes surface white, pale tan to brownish, mottled dark brown with pale areas when handled and dried, smooth when fresh, much wrinkled when dried, with some white mycelial strands, soil, and organic matter adherent to surface. Peridium <0.5 mm wide, somewhat separable. Hymeneal gleba olive green when young, green-brown to dark brown when mature, nearly black when dried, locules ellipsoid to elongate, stuffed with spores, columella absent when young, thin dendroid in mature, gelatinized, grayish. Rhizomorphs few, small, white attached at base, brownish when handled or when herborized. Odor organic solvent-like. Taste not recorded. **Macrochemical characters.** Positive reaction with KOH (5%), brown to blackish on the surface of dried specimens.

Micromorphology. Peridium 70-400 μ m wide, two-layered. Epicutis 45-175 μ m wide, usually on the thinner side with some areas with wart-like protrusions, of septate hyphae, thin-walled, pale yellow-brown to yellow-brown in KOH, repent hyphae 4.5-6.5 μ m broad, occasionally inflated cells up to 18 μ m broad, with interspersed small crystalline particles scattered across the layer, subcutis 110-135 μ m wide, of septate, thin-walled, hyaline in KOH, interwoven to subparallel or cross-weaved hyphae, 6.5-11.0 (-15.0) μ m wide. Mycelial strands on peridium of dark brown, filiform, branched hyphae, 2-3 μ m broad, encrusted with small crystalline particles, clamp connection present. Trama 37-112 μ m wide, hyaline in KOH, thin-walled, compactly interwoven to parallel hyphae, 2-5 μ m wide, in a gelatinized matrix, clamp connections present. Basidia not observed. Spores without utricles and hilar appendage 10.5-12.3 x 5.3-7.0 μ m, mean = 11.3 x 6.0 μ m, with utricles and hilar appendage (12.3-) 13.2-14.0 x 7.9-9.7 (-10.5) μ m, walls up to 1 μ m thick, oblong fusoid, ellipsoid, symmetrical, smooth when young, spinny within the utricles when mature and not encompassing hilar appendage, often not equally inflated, rarely encompassing entire spore, commonly laterally inflated

up to 5 (-6) μm broad, hyaline to yellow-orange singly, pale brown in mass in KOH, inamyloid, non-dextrinoid in Meltzer reagent.

b) *Distribution, habit, habitat, and ecology*

México, Michoacán, in the Trans-Mexican Volcanic belt, hypogeous, solitary to groups, under *Quercus castanea* Muhl. *Q. obtusata* Bonpl., *Q. magnoliifolia* Née, *Q. rugosa* Née, *Pinus leiophylla*, *Pinus pseudostrobus* Schl. & Cham., and *Pinus michoacana* Mtz. at approximately 2160 m elevation, September and October.

c) *Specimens examined*

State of Michoacán, MÉXICO, locality Puerto Madroño; ejido Atécuaro, Municipality of Morelia, 19° 32' 113", 101° 12' 5", 18 Oct. 2011, G Guevara 1211 (ITCV 1211 holotype), G. Guevara 1225 (ITCV 1225 paratype), G. Guevara 1218 (ITCV 1218). Ichaqueo, 20 Sept. 2014, V Gomez-Reyes 863, 877, (EBUM paratype).

Observations. *Pterosporomyces herrerae* is recognized morphologically by its olive-green to the brown-green color of the gleba and spores with a distinctive inflated, wing-like appearance to the utricle (inflated up to 6 μm), associated with *Quercus* spp. and ITS, ATP6 gene variation. *Restingomyces reticulatus* is similar to *P. herrerae*, but the former has reticulated basidiospores. This novel taxon also resembles to *Hysterangium inflatum* Roadway, a member in the family Hysterangiaceae with olive-green, brown to black gleba but differs by presenting smaller spores of 9-12 x 8-9 μm including ornamentation with utricle up to 2.5 μm on the side, ectomycorrhizal associated with *Eucalyptus* spp. and very distant genetically in ITS and ATP genes. Similarly, *Hysterangium stoloniferum* Tul. & C. Tul. resembles *Pterosporomyces herrerae*, but the former presents bigger spores of 17-21 x 6-8 μm and a narrow adnate rugose utricle.

Discussion. The study of hypogeous fungi has been very limited in Mexico (Cázares et al. 1992; Trappe & Guzman 1971). However, in 2016 *Aroromyces balanosporus* and *A. herrerae* were proposed as new taxa based only in macro (brown to blackish gleba) and microscopic (fine spines within the utricle) morphology without molecular support (Guevara et al. 2016; Castellano et al. 2000). Posteriorly, an ATP6 and ITS gene analysis were performed on these species to confirmed their novel status. The analysis confirmed the novel status of *A. balanosporus* (Genbank MK811031) as expected (Peña-Ramirez et al. 2019). However, the phylogenetic study surprisingly revealed that *A. herrerae* belongs to the evolutive basal family Trappeaceae and *Trappea darkeri*, *Restingomyces reticulatus*, and *Phallobatia alba*. Thus, *Aroromyces herrerae* was transferred from the Hysterangiaceae to *Pterosporomyces* within the family Trappeaceae, an ancestral taxon in Phallales. The basidiomata of

Pterosporomyces herrerae in cross-section are similar to those in *Restingomyces reticulatus* having both brown jelly dendroid gleba as seen in *Aroromyces*, but differs in the reticulate spore ornamentation present in *Restingomyces*; in contrast, *Pterosporomyces herrerae* shows utricle (wing) spores up to 5(6) μm tall. It seems to be that the utricle is a convergent evolutive feature similarly observed in other hypogeous species such as *Austrogautieria* in the family Gallaceaceae, which possesses longitudinally ridged spores alike to those in *Gautieria* (Hosaka et al. 2006). Similarly, the ITS and ATP6 genes analysis showed that *Pterosporomyces herrerae* along with *Restingomyces reticulatus* is ancestral of epigeous related genera such as *Clathrus*, *Phallus*, *Dictyophora*, *Mutinus*, among other stinkhorn taxa that evolved from hypogeous gasteroid forms in the Phallales clade in agreement with Hosaka et al. (2006) and Sulzbacher et al. (2016). Fig. 1 & 2.

With these results, the members of the family Trappeaceae increase to five, *Trappea darkeri*, *T. phillipsii*, *T. pinyonensis*, *Restingomyces reticulatus*, and *Pterosporomyces herrerae*, hoping to answer the question, "how many more taxa await to be described from the Neotropical Forest?"

ACKNOWLEDGEMENTS

Guevara acknowledges TecNM (Tecnológico Nacional de México), SES-PROMEP, CONACYT and Kelly L. Forte for research and editorial support. M.A Montalvo thanks CONACYT for economic scholarship. Z-WG was supported by the National Natural Science Foundation of China (No. 31872619).

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