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A new species of *Phlebopus* (Boletales, Basidiomycota) from Mexico

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Abstract: A new species, *Phlebopus mexicanus,* is described from southern tropical rainforests of Mexico based on morphological and molecular characters. Several features distinguish this species from others of *Phlebopus* including the medium to small basidiomata with olivaceous brown tomentose pileus that becomes finely areolate cracked with age, the dark yellow brown pruina covering most of the stipe, the pale yellow flesh of pileus and stipe that slowly turns blue when exposed, and the lack of hymenial cystidia. Phylogenetic analyses using nLSU sequences support the recognition of this new morphological species in the Sclerodermatineae. Our analyses also suggest that *P. portentosus* and *P. marginatus* are not conspecific and relationships of Old World taxa of *Phlebopus* need further scrutiny. A key to all known New World taxa is provided.

Key words: boletes, identification key, phylogenetics, Sclerodermatineae.

Introduction: *Phlebopus* (R. Heim) Singer is a genus of tropical boletes characterized by basidiomata with typically robust stature with a central thick, non-hollow stipe, short smooth olivaceous brown basidiospores, and abundant clamp connections on hyphae of the basidiomata (Singer 1986, Watling 2008). There are currently 21 taxon names listed under *Phlebopus* in the Index Fungorum website (http://www.indexfungorum.org/Index.htm), of which 14 are potential members of the genus. *Phlebopus* species are described and reported from tropical and subtropical areas of Central and South America, Africa, Asia and Australia (McNabb 1968, Heinemann and Rammeloo 1982, Singer et al. 1983, Watling and Gregory 1988, Watling and Turnbull 1992, Li and Watling 1999, Pham et al. 2012). In 1982 Heinemann and Rammeloo provided a global survey of *Phlebopus*. They described, discussed and provided a key to twelve species including three occurring in the Americas, namely *P. beniensis* (Singer & Digilio) Heinem. & Rammeloo, *P. bruchii* (Speg.) Heinem. & Rammeloo and *P. tropicus* (Rick) Heinem. & Rammeloo.

Singer et al. (1983), in a survey of ectotrophically mycorrhizal fungi of the neotropics, published a key to 11 taxa of *Phlebopus* including the new species, *P. brasiliensis* Singer. They provided limited descriptions and/or discussions for most of the taxa included in their paper, except for *P. beniensis* which was studied in detail. Apparently they were not aware of Heinemann and Rammeloo's (1982) publication since they made no reference to it. In 1986 Singer listed ten species in the genus and synonymized *P. bruchii* and *P. harleyi* Heinem. & Rammeloo with *P. braunii* (Bres.) Heinem.

In the Americas, *P. beniensis*, originally described from Bolivia (Singer and Digilio 1960), seems to be the most common and wide spread species since it is reported from Argentina, Bolivia, Brazil, Costa Rica, Ecuador, Martinique, Puerto Rico and Venezuela by Heinemann and Rammeloo (1982), Ovrebo (1983), Pegler (1983), Singer et al. (1983, 1990), Miller et al. (2000) and Guzmán et al. (2007). Of the remaining three taxa of *Phlebopus* described from South America, little is known. Deschamps and Moreno (1999) provide a modern description of *P. bruchii* noting its widespread occurrence and food use by indigenous peoples in northern Argentina. Descriptions of *P. beniensis* and *P. brasiliensis* are available in Singer et al. (1983) as well as a key with some descriptive information for each taxon. Modern descriptions for *P. tropicus* are lacking.

In Mexico, there are three species of *Phlebopus*. Singer et al. (1990) and Bandala et al. (2004) reported on *P. portentosus* (Berk. & Broome) Boedijn, Guzmán and Guzmán-Dávalos (1984) and Cifuentes (2008) on *P. colossus* (Heim) Singer from tropical zones near Cotaxtla and Uxpanapa, and García-Jiménez and Garza-Ocañas (2001) on *P. brasiliensis*. Based on these publications, descriptions of the Mexican material of *P. portentosus* are available but not of *P. colossus* and *P. brasiliensis*. Previously, *P. portentosus* and *P. colossus*, taxa producing very large basidiomata, were known from the Old World tropical regions (Sri Lanka, Madagascar, Australia, Africa, and China). *Phlebopus portentosus* is considered by some authors to be a synonym of *Phlebopus marginatus* Watling & N.M. Greg., originally described from Australia (Watling and Gregory 1988, Lei et al. 2009).

The biology of *Phlebopus* species is not well understood, but at least some species may be ectomycorrhizal (Singer, 1986; Binder and Hibbett, 2006, supplement) and a few form an unusual relationship with scale insects that appear to be plant root pathogens (Singer, 1986, Pham et al. 2012). It is not clear from our field observations if the new species we present here is ectotrophic or saprotrophic or plays some other ecological role.

Recent molecular studies place *Phlebopus* in the order Boletales, suborder Sclerodermatineae, family Boletinellaceae, and closely related to the genus *Boletinellus* (Binder and Hibbett 2006, Wilson et al. 2011). Phylogenetic analysis based on nuclear LSU (nLSU) sequences was used to evaluate the placement of the new species among members of the suborder Sclerodermatineae. A detailed morphological description of the new species *P. mexicanus* is presented as well as a key to the seven species of *Phlebopus* currently reported from the Americas.

Materials and Methods: Herbarium abbreviations follow Thiers (2014). Color notations in parentheses come from three different sources. Those designated as number/letter/number (e.g. 5D5) are from Kornerup and Wanscher (1978) and can be an exact color hue like Clay (5D5 – Clay) or a general color indicated by the lower case script (e.g. 3A2 – yellowish white). Codes as letter/number lower case/ letter/number lower case/ letter/number lower case (e.g. are from Küppers (1994) and those as e.g. 2.5Y8/2 are from Munsell (1998). Microscopic features were studied in 3% KOH, 10% NH_4OH , Congo Red in 10% NH_4OH, and/or Melzer's Reagent (Smith and Thiers, 1971). For basidiospore measurements, the hilar appendix or apiculus was excluded. In those measurements Q refers to the length divided by the width of an individual spore. The notation n = 41/2, means that 41 individual basidiospores were measured from 2 different collections. Means for length and width are given with their standard deviations, as is the mean of Q values, with the mean of the length divided by the width of all basidiospores measured being designated as Q^m. Descriptive statistical analysis of basidiospores was developed using EXCEL for MAC 2011. All light microscopic images were made with an Olympus BX 50 transmitted light microscope using DIC or bright field optics and captured with a Diagnostic Instruments, Inc. Insight Spot 3-shot color digital camera.

DNA sequences of the ITS (ITS1, 5.8, ITS2) and 5' end of the nLSU regions of *P. mexicanus* were obtained in this study. DNA extraction, amplification and sequencing from dried specimens of *P. mexicanus* were performed at the Center for Forest Mycology Research in Madison following Palmer et al. (2008). The ITS region was amplified with primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) while the 5' end of the nLSU region was amplified with primers LROR and LR5 (Vilgalys and Hester 1990). DNA sequences were used primarily for molecular identification and were compared with other sequences available in GenBank via BLAST search. nLSU sequences were also used to infer the phylogenetic relationship among *P. mexicanus* and other members of the suborder Sclerodermatineae (Fig. 1).

A total of 26 nLSU sequences were retrieved from GenBank (Benson et al. 2011), including five species of *Phlebopus* (*P. beniensis*, *P. marginatus, P. portentosus*, *P. spongiosus* and *P. sudanicus*) and at least one species of the genera *Astraeus*, *Boletinellus*, *Calostoma*, *Diplocystis*, *Gyroporus*, *Pisolithus*, *Scleroderma* and *Veligaster*. *Suillus hirtellus* (Peck) Snell and *Suillus punctipes* (Peck) Singer (suborder Suillineae) were used as outgroup taxa in the phylogenetic analyses. Sequences were edited with Sequencher 4.8 (Gene Codes Corp., Ann Arbor, Michigan), and aligned using the G-INS- I algorithm in MAFFT v.6 (Katoh and Toh 2008). The alignment was manually adjusted using MacClade 4.08 (Maddison and Maddison, 2002). The LSU dataset was compiled and evaluated with two phylogenetic analyses: (i) Maximum likelihood analysis (ML) run in the RAxML server, v.7.2.8. (Stamatakis et al. 2008) under a GTR model with 100 rapid bootstrap replicates, (ii) equally weighted parsimony analysis (MP) performed using PAUP* v.4.0.b10 (Swofford 2002) with 1000 heuristic search replicates performed with starting trees generated by stepwise addition with random additions sequences followed by tree bisection

reconnection branch swapping and up to two trees kept in each replicate, with bootstrap analysis estimated from 1000 replicates with 10 random taxon addition sequences. Sequences generated in the present study were deposited in GenBank (accession numbers KM675999-KM676002).

Results:

Taxonomy

Phlebopus mexicanus Cifuentes, Cappello, T. J. Baroni & B. Ortiz, sp. nov. Figs. 2-6
MYCOBANK #: MB 811065

Diagnosis. A species of *Phlebopus* distinguished by the small to medium sized stature, olivaceous, tomentose pileus becoming finely areolate at least over the disc, pale yellow or yellowish brown stipe heavily covered below the glabrous apex with yellow brown granulose pruina, flesh in the pileus and stipe yellow but slowly turning blue, basidiospores short ellipsoid, averaging 6.4 x 5 µm, absence of cystidia in the hymenium, and abundant clamp connections on all hyphae. With LSU sequences supporting the lineage as distinct from all other sequenced taxa.

Holotype. MEXICO. Tabasco, Centro County, Jardines Campus DACB UJAT (17° 59' 24.35" N 92°58' 25.85" W), terricolous, under *Salix* sp., 20 October 2009, J Cifuentes and S Cappello 2009-233 (FCME, isotype UJAT).

Pileus yellowish café (N10 Y90 M20) or café olivaceous (2.5Y3/3) or yellowish brown (5E3-4: Mouse Grey or Hair Brown) when young, developing olivaceous hues with age (M30 Y80 Y90 M20 M30), drying to a paler grayish yellow (4B4: Champagne), ground color between areolae yellowish (2.5Y8/2) or some with reddish café discoloration, 65-95 mm broad, convex becoming plane, margin decurved, surface dry, superficially smooth at first, but velvety granulose under a lens, disc more distinctly granulose, tomentose or velvety and with expansion becoming areolate. **Context** fleshy, very pale yellowish cream (3A2 – yellowish white) or grayish yellow (3C3: Ash Blonde) and becoming slowly and lightly blue or bluish green, then intense blue (Y50 M40 C80) or dull green (25D4) especially near the tubes, 15 mm deep. **Pores** 1-2 per mm, angular or polygonal, irregular or radially arranged, grayish yellow at first (4C3-4: Beige, Blonde), becoming yellowish green or more olive brownish (4D5 - Khaki) (Y90M30C30), in young specimens turning very slowly blue or bluish green and then slowly turning reddish brown when bruised. **Tubes** concolorous with pores or lighter in color (3B4: Straw yellow), when cut open slowly developing some bluish or bluish-green discoloration, 8 mm long, adnate or shallowly adnexed (shallowly depressed) or free. **Stipe** pale yellowish over apex (3-4A4 – pastel yellow, light yellow) (N00Y90M00), downward progressively darker and thus concolorous with pileus or yellowish brown (5D5 - Clay), becoming darker brown over the base (7B-F4), becoming brown (6E5) in the paler yellow brown areas when handled, 10-15 mm broad at apex, 20-50 mm long, cylindrical or subclavate, smooth over apex, and yellowish brown (5D5 - Clay) granulose-pruinate to base, flesh very pale yellowish cream (3A2 – yellowish white) when first exposed, slowly developing blue or blue green or greenish colors as the pileus context. **Odor** mushroom-like. **Taste** somewhat sweet. **Spore Print Color:** not obtained**.**

Basidiospores 5.5–7.5 × 4.5–5.6 µm (n = 41/2, $\bar{x}$ = 6.4 ± 0.6 × 5.0 ± 04, Q = 1.1–1.6, Q^m = 1.3 ± 0.1), short ellipsoid, smooth, ± thick-walled (approx. 0.5 µm), dark olive brown in 3% KOH, often with one large guttule. **Basidia** (1-2)-4 sterigmate, broadly clavate, 17.8–28.3 x 7.2–8.8 µm. **Hymenial cystidia** absent. **Tube trama** hyaline with a golden or yellowish refractive narrow mediostratum, hyphae parallel or very slightly divergent in the laterstratum, slightly gelatinized in the laterostratum with glassy undulate collapsing hyphae, 6.4–10.5 µm in diam

and loosely interwoven, mediostratum hyphae 1.6–3.2 µm in diam and tightly parallel. **Pileus context** hyaline, loosely interwoven, very thin-walled and often collapsing, cylindrical or inflated, 4–16.2 µm in diam. **Pileipellis** a dark brown layer of tufted, pigmented trichodermial hyphae interspersed with hyaline interwoven mostly repent hyphae, 4–7.2 µm in diam, end cells mostly cylindrical, pigmented ascendent tufts of hyphae with dark yellow brown intracellular amorphous pigments also encrusted with very dark reddish brown shiny pigments in dH_2O, these encrusting pigments dissolving in 3% KOH and 10% ammonia producing a diffuse brownish pigment that quickly dissipates. **Stipitipellis** ± hyaline or pigmented as pileus surface, the pruinate areas composed of a trichodermial layer of multi-septate and heavily clamped hyphal cells, 2.4–8 µm in diam, with versiform caulocystidiate end cells, these often clavate, fusoid, ventricose, narrowly cylindrical, walls often undulate, 17.8–40.5 x 4–7.2 µm. **Clamp connections** present on all hyphae and obvious.

Habit, habitat, fruiting period: Gregarious, on duff and soil, in tropical rainforest and deciduous forest and also in tropical gardens, from August to October.

Distribution: Southern Mexico. See Fig. 7 for distribution map.
Additional specimens examined: MEXICO. Campeche, Hopelchén County, Tah-Cok Archaeological Site (19.7444°N 89.8453°W), near *Brosimum alicastrum* Sw., 7 October 2012, Cifuentes and Vázquez-Estup 2012-275. Tabasco, Centro County, Gardens Campus DACB UJAT (17° 59´24.35" N 92° 58´25.85" W), under *Salix humboldtiana* Wild, 11 June 2008, S. Cappello 2412, 29 June 2009, S Cappello 2507, 9 September 2012, S. Cappello 2688; Cunduacan County, Cunduacan (18° 03´42.72" N 93° 09´50.17" W), under *Erythrina americana* Mild., 19 September 2008, S. Cappello 2451; Macuspana County, Road to Chivalito, Apasco near Cascadas de Agua Blanca (17° 37´ 20.53" N 92° 26´ 51.64"W), under *Brosimum alicastrum* Sw., 3 August 2012, S. Cappello 2806, Limbano and Blandin town (17° 44' 59.60" N 92° 23' 27.00" W,), under *Coccoloba barbadensis* Jacq., 19 September 2008, S. Cappello and V.H. García 1909 2008-1.

Phylogenetic analyses

The phylogenetic relationship among *Phlebopus* and other members of suborder Sclerodermatineae was inferred using nLSU sequences and two phylogenetic analyses (ML and MP). Results are based on the topology of the best tree from the ML analysis. The nLSU dataset included 28 ingroup sequences and 903 characters, of which 180 were parsimony informative and 668 were constant. Members of the suborder Sclerodermatineae were grouped into three moderate to strongly supported clades: clade 1 including species of *Astraeus*, *Diplocystis*, *Calostoma*, *Gyroporus*, *Veligaster*, *Scleroderma* and *Psilothus*; clade 2 including *Boletinellus* species and clade 3 including *Phlebopus* species. Sequences of *Phlebopus mexicanus* grouped with other *Phlebopus* species, forming a monophyletic clade (Fig. 1).

Discussion: The analysis of nLSU DNA sequences of *Phlebopus mexicanus* (Fig. 1) placed it within the *Phlebopus* clade, confirming its generic placement among members of the Sclerodermatineae. nLSU and ITS sequences compared via a GenBank BLAST search indicated that *P. mexicanus* is related to isolates of *P. marginatus* (100% query cover and 97% identity with nLSU sequences of isolates MEL2145841 and REH 8883; and 99% query cover and 81% identity with the ITS sequence of isolate REH 8883). The tree topology obtained in the present study using the nLSU DNA sequences of *Phlebopus* available in GenBank, also provides additional information to be consider in further studies of the genus: (1) sequences of *P. portentosus* and *P. aff. portentosus* do not group

in the same clade, (2) isolates of *P. marginatus* do not appear on the same clade with isolates of *P. portentosus*, suggesting that these taxa are not conspecific or the taxa are misidentified, and (3) isolates of *P. portentosus* and *P. sudanicus* clustered on the same clade, suggesting that they are conspecific.

The combination of distinctive macroscopic features, microscopic features and the molecular data support the recognition of a new neotropical species of *Phlebopus*, *P. mexicanus*. *Phlebopus mexicanus* is small in stature, but its distinctive short olivaceous brown basidiospores, abundantly clamped hyphae, and the lack of a distinctive ectotrophic partner, indicates it is a species of *Phlebopus*. *Phlebopus portentosus* and *P. colossus*, previously reported from Mexico, are very large species that do not compare morphologically to *P. mexicanus*. The bluing reaction of the flesh is similar to the much larger neotropical *P. beniensis* described from northern South America (Bolivia and Argentina), but *P. mexicanus* differs from *P. beniensis* by several morphological features in addition to its size. For example, *P. beniensis* (as described by Singer et al. 1983) produces a smooth, pale umber, sometimes viscid pileus that lacks olivaceous hues, while *P. mexicanus* has a pileus that is dry, distinctly granulose, velvety or tomentose that becomes areolate, is olivaceous in color with yellow ground color between areolae and develops reddish hues with age that remain on the dried pileus. In addition, *P. beniensis* has a subglabrous stipe that is cream over the apex but fuliginous from the base upwards, while *P. mexicanus* is roughly pruinate overall with a pastel yellowish apex and olive or yellowish brown elsewhere with distinctive yellowish brown granulose pruina overall. The basidiospores of *P. beniensis,* 5.8–7.5 x 4.3–6.2 µm, are similar in size with *P. mexicanus*, however, *P. beniensis* has abundant hymenial cystidia, especially on the pore edges, whereas *P. mexicanus* lacks hymenial cystidia.

Phlebopus brasiliensis is comparable in size with *P. mexicanus* with a pileus 30–65 mm broad and stipe 35–40 mm long and 11 mm wide at the apex (Singer et al. 1983). These two species differ in a number of features, however. The main difference is that the yellowish flesh of *P. brasiliensis* does not turn blue on exposure to air as in *P. mexicanus*. The pileus of *P. brasiliensis* is velvety or somewhat tomentose but smooth while the stipe is glabrous and smooth. These macrofeatures are in sharp contrast *to P.* mexicanus which has a distinctly tomentose pileus that becomes areolate and the stipe is heavily ornamented with granulose pruina. Hymenial cystidia are present but not numerous for *P. brasiliensis* and lacking in *P. mexicanus*.

Key to Phlebopus species reported in the Americas

Phlebopus is characterized by basidiomata ranging from medium to very large size with a central thick, non-hollow stipe, pores mostly small, basidiospores short smooth olivaceous brown, hymenial cystidia present or inconspicuous or absent, and clamp connections usually present on hyphae of the basidiomata. A small genus with a pan-tropical distribution, broader in the Southern Hemisphere.

1. Pileus large (more than 100 mm broad) 2
1. Pileus small to medium (less than 100 mm broad) 3

2. Pileus mostly black, lacking hymenial cystidia ..... ***P. colossus***
2. Pileus mostly brown with olive tints, hymenial cystidia present .....***P. portentosus***

3. Pileus ochre or yellowish brown with olivaceous tones 4
3. Pileus reddish brown or dark brown without olivaceous tones 5

4. Pores wide or large (1 mm or more in diam.) ..... ***P. tropicus***
4. Pores smaller 6

5. Context bluing when bruised (at least on the stipe) ..... ***P. bruchii***
5. Context not bluing when bruised ..... ***P. brasiliensis***

6. Hymenial cystidia present ..... ***P. beniensis***
6. Hymenial cystidia absent ..... ***P. mexicanus***

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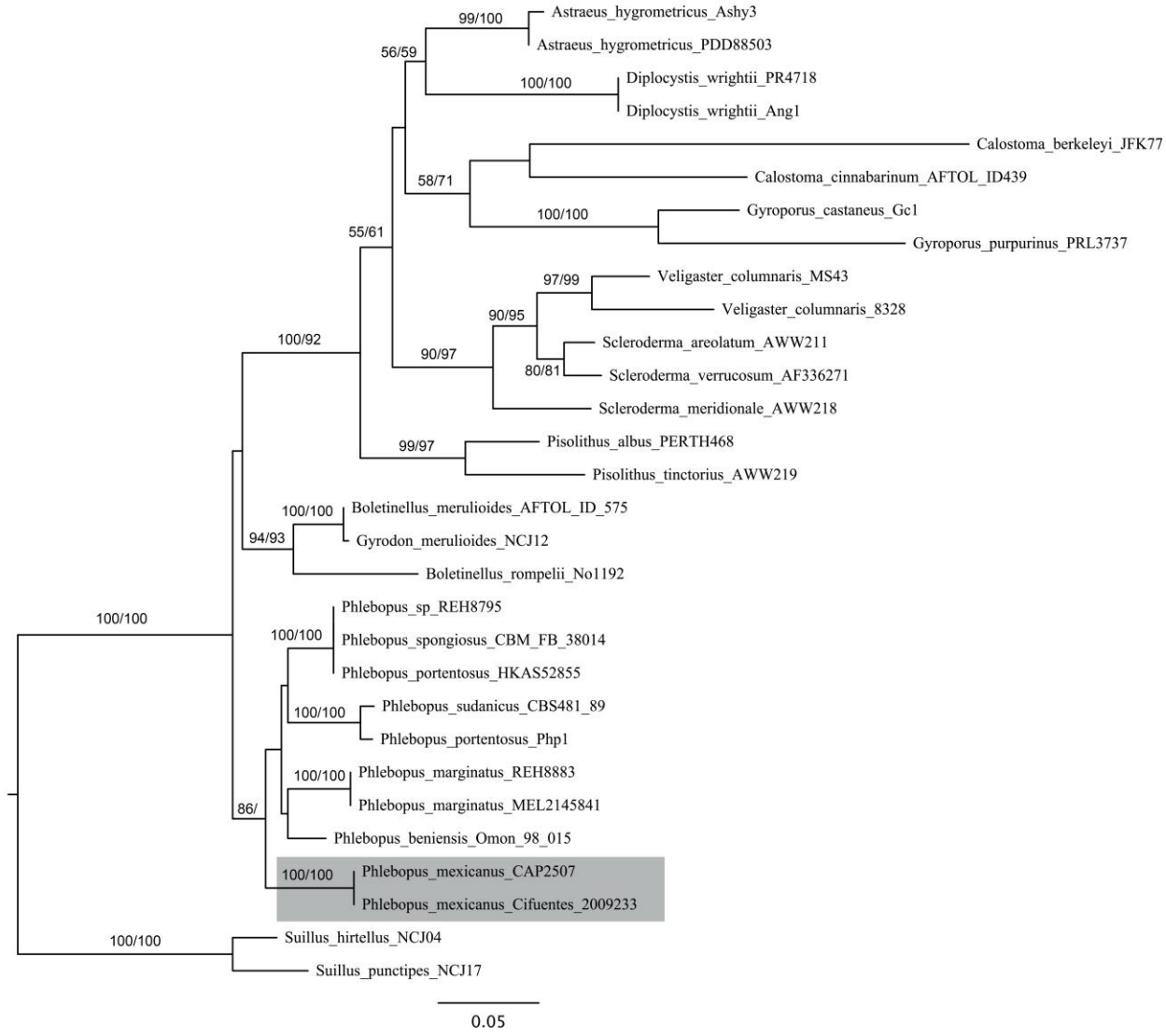
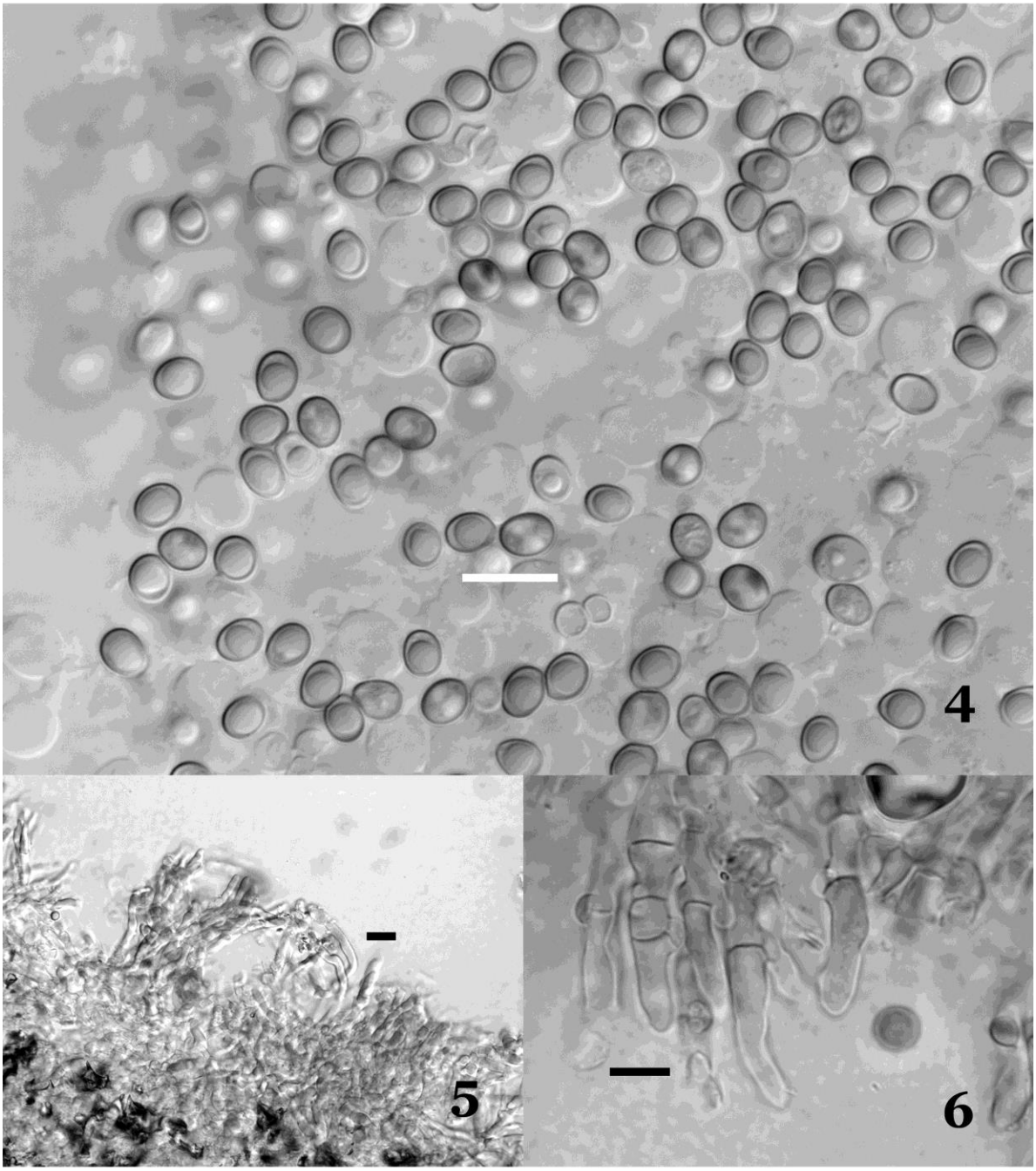


Figure 1. Phylogenetic relationships of members of the family Boletinellaceae inferred from the analysis of nLSU rDNA sequences. Topology from maximum likelihood analysis. Support values along branches are from maximum likelihood bootstrap (≥70) and maximum parsimony bootstrap (≥50) respectively.

Figures 2-3. Fresh basidiomata of *Phlebopus mexicanus*, Cifuentes 2009-233 (Holotype). Fig. 1. Basidiomata intact. Fig. 2. Same specimens as in Fig. 2 but one basidiome cut in half to show the context and the faint bluing reaction.



Figures 4-6. Transmitted light photomicrographs of microscopic structures of the basidioma. Fig. 4. Basidiospores of *Phlebopus mexicanus*, Cifuentes 2009-233 (Holotype). Fig. 5. Pileipellis of *Phlebopus mexicanus*, Cappello 2507. Fig. 6. Stipitipellis with multiseptate caulocystidia with clamp connections on the septa in *Phlebopus mexicanus*. Cappello 2507. All scale bars = 10 µm.

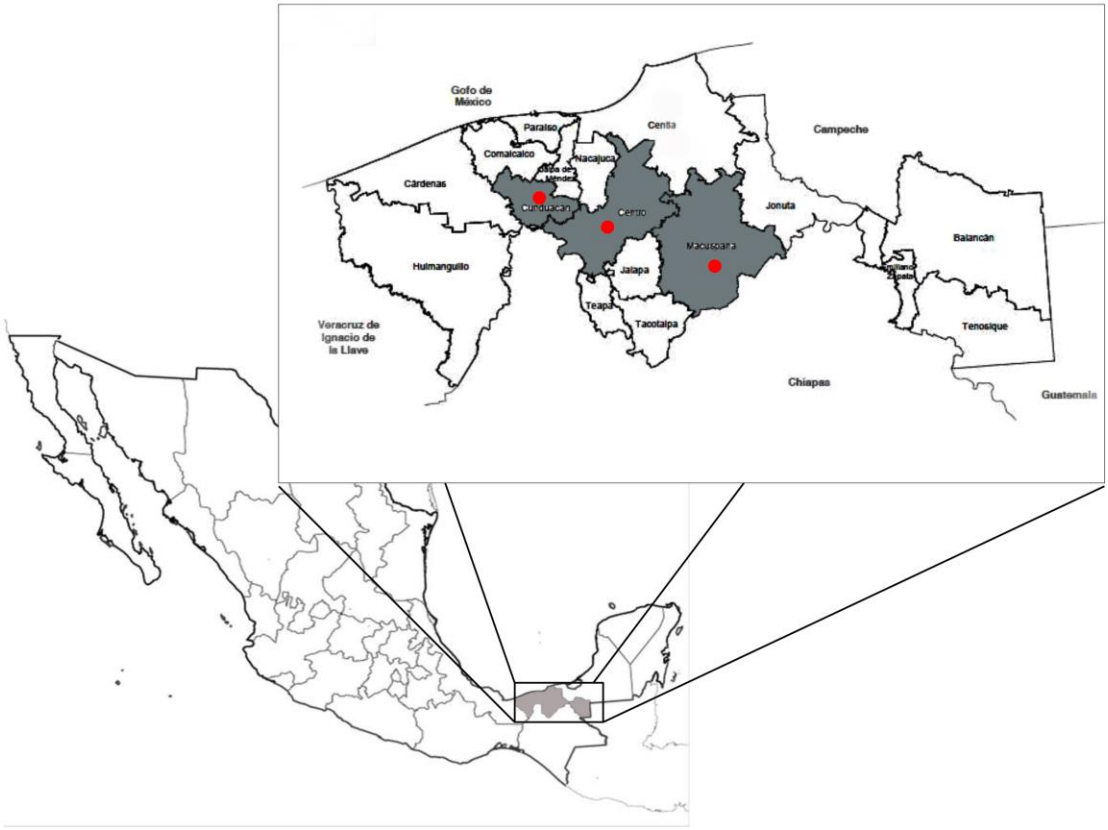


Figure 7. Distribution of collections in the counties of Campeche and Tabasco States.