

Morphological, Molecular, and Phylogenetic Studies of *Amanita brunnescens* from the Cuenca de la Esperanza Protected Natural Area, Guanajuato, Mexico

Juan Manuel Alejandro Aguiñaga Pacheco¹, Gonzalo Guevara Guerrero¹, Rafael Peña Ramírez², Noemi Garcia Alcaraz¹, Maricarmen Islas Peralta¹ and Alfredo Heriberto Herrera Estrella³

¹ División de Estudios de Posgrado e Investigación. Instituto Tecnológico de Ciudad Victoria. Boulevard Emilio Portes Gil, no. 1301, Ciudad Victoria, Tamaulipas, México.

² Instituto Tecnológico Superior de Irapuato, Carretera Irapuato-Silao km 12.5, Irapuato, Guanajuato, México.

³ Centro de Investigación y de Estudios Avanzados del IPN, Unidad Irapuato (Cinvestav), Libramiento Norte Km. 9.6 Irapuato, Guanajuato, México.

E-mail: gonzalo.gg@cdvictoria.tecnm.mx

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Abstract: The study of macrofungi, especially the genus *Amanita*, is extensive and well-documented. In Mexico, a wide variety of edible and poisonous species is known, underscoring the importance of studying them. In this study, we examined a series of collections from central Mexico whose genetic identification matched *Amanita brunnescens*, a species native to eastern North America. This species is primarily associated with hardwood forests and some conifers and is characterized by its dark brown to dull brown coloration. However, in the collections from this research, we observed morphological variation that does not fully align with the characteristics described by the original author of the species. This led us to question whether these variable traits correspond to a single species or a species complex within this taxon. To test these hypotheses, we performed maximum-likelihood and Bayesian phylogenetic analyses, which, together with the ASAP and GMYC species delimitation methods, suggest that these morphological differences are likely due to diversification.

Keywords: Amanitaceae, Basidiomycetes, biocultural, taxonomy, toxic.

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Introduction

Amanitaceae E.-J. Gilbert is a family with cosmopolitan distribution (Bas 1969; Cui et al. 2018; Zhou et al. 2023). The main genus *Amanita* Persoon (1797) comprises 95% of the species in the family and includes ectomycorrhizal species as well as some saprotrophs (Redhead et al. 2016; Jabeen et al. 2019). The classification proposed by Bas (1969), Corner and Bas (1962) divides the genus into two subgenera based on their morphology: *Amanita*, with three sections, and *Lepidella*, with four sections,

using morphological characters to separate them. The authors Imazeki and Hongo (1987), Jenkins (1977, 1986), Mao (1990), Pegler and Shah-Smith (1997), and Tulloss *et al.* (1992, 1995) supported this classification.

Whereas Cui *et al.* (2018) established three subgenera based on molecular support: *Amanita* with four sections, *Amanitina* with six sections, and *Lepidella* with one section, inferred from the markers nrLSU, rpb2, tef1- α , and β -tubulin, Huang *et al.* (2023), Kumar *et al.* (2021), Liu *et al.* (2022), and Zhou *et al.* (2023) accepted and reinforced this classification in their studies.

The Sect. *Validae* (Fr.) Quél. within the family, according to the classification of Cui *et al.* (2018), in the subgenus *Amanitina* E.-J. Gilbert (1940) is characterized by the absence or partial presence of veil remnants on the pileus, a sometimes poorly defined cuticle, often white to pale, the margin generally covering the lamellae and appendiculate, a membranous or thick and disruptive ring, or brittle, leaving the stipe flocculose-pulverulent. The stipe often has a large bulb, which may be marginate or not; the bulbous base may be absent or present. Spores globose to subcylindrical (Corner and Bas 1962; Cui *et al.* 2018).

Amanita brunnescens, described by George Francis Atkinson as a common species in eastern North America in oak and conifer forests, was previously interpreted as *A. phalloides* with dark brown coloration; the author himself refuted this comparison (Atkinson 1918). *Amanita brunnescens* var. *pallida*, described by Krieger (1927), shares all the characteristics of Atkinson's species (1918), except for its pale coloration (Krieger 1927).

In Mexico, studies on this genus have been carried out, reporting some edible species, e.g., *Amanita basii* Guzmán and Ram.-Guill., *A. jacksonii* Pomerl., *A. laurae* Guzmán and Ram.-Guill., and *A. rubescens* Pers. (Guzmán 1973; Acosta and Guzmán 1984; Pérez-Moreno and Ferrera-Cerrato 1995; Guzmán and Ramírez-Guillén 2001; Hernández-Rico and Moreno-Fuentes 2010; Garza Ocañas *et al.* 2023). These species have great biocultural potential due to their nutritional and economic impact, as well as their sustainable use by rural communities (Ruan-Soto *et al.* 2004; Estrada-Martínez *et al.* 2009; Torres-Gómez *et al.* 2023). On the other hand, some poisonous species have also been reported, e.g., *A. verna* Bull. ex Lam., *A. bisporigera* G.F. Atk., *A. ocreata* Peck, *A. porphyria* Alb. & Schwein., *A. aspera* var. *aspera* (Pers.) Pers., *A. virosa* Secr., *A. flavoconia* G.F. Atk., and *A. pantherina* (DC.) Krombh. (Guzmán 1973; Pérez-Silva and Fuentes 1982; Pérez-Silva and Herrera 1982; Acosta and Guzmán 1984; Pérez-Moreno and Ferrera-Cerrato 1995; Hernández-Rico and Moreno-Fuentes 2010; Garza Ocañas *et al.* 2023), some of which have also been observed in Guanajuato as part of the local mycobiota.

In this work, collections made in the state of Guanajuato are examined and, using molecular markers from the ITS and EF1 regions, are related to the North American species *A. brunnescens*. These samples share morphological characteristics expected of the species, e.g., a bulbous stipe, veil remnants on the pileus, and yellowish-cream, brown, or pale coloration. However, they differ in other aspects, such as the presence, absence, or shape of an annulus, striated margins, and a sacciform volva.

The morphological inconsistencies and their apparent genetic relationship with the species led us to study the phylogeny of these two regions by constructing Maximum Likelihood (ML) and Bayesian Inference (BI) trees of the concatenated and individual sequences (ITS and EF1), as well as the application of the species delimitation methods Assemble Species by Automatic Partitioning (ASAP) and General Mixed Yule Coalescent (GMYC) as tools to understand the status of the species found. Determining these

characteristics helped us assess whether the morphological variations were attributable to a single species or a complex within the epithet *brunnescens*.

Materials and methods

Study area

The study was conducted in the protected natural area Cuenca de la Esperanza "Las Palomas" in Guanajuato, composed mostly of oak forest, with associations of *Quercus* sp., *Arbutus* sp., and *Arctostaphylos pungens* (Doniz-Padilla et al. 2010).

Collection method and morphology

Specimens were collected using the standard methodology for Agaricales studies described by Largent and Stuntz (1977), Largent and Thiers (1977), Largent et al. (1977), Lodge et al. (2004), and C. Bas (1969). Macroscopic characters were described from fresh material, photographs, and observations of dried material. Microscopic structures were observed with an optical microscope (B-600T; Optika, Ponteranica, Italy) using the 4×/0.10, 10×/0.25, 40×/0.65, and 100×/1.25 oil-immersion objectives. Cross sections of the trama, lamellae, stipe, and pileus were mounted in 5% KOH and Melzer's reagent (Largent et al. 1973). Spores were measured with a micrometer; 50 spores were examined. Size and shape ranges were obtained measuring the width and length measurements (Q) to establish ranges for both, using typical minimum and maximum values and discarding outliers. Biometric variables for basidiospores were obtained from 50 mature spores [50/1/1], measuring length and width to calculate the length/width quotient (Q) and the mean quotient (Qm). Biometric ranges are presented as (a–)b–c(–d), where b–c contains at least 90% of the measured values and extreme values are given in parentheses. Basidia were measured for length, width and sterigma length. Marginal cells of the lamellar edge were also described. The bilateral lamellar trama was characterized based on the type, arrangement, and element composition of the mediostratum, lateral stratum, and subhymenium, the ranges and measurements were obtained using the method described by C. Bas (1969),

DNA extraction, amplification, and sequencing

DNA was extracted from dried tissue using the CTAB method of Möller et al. (1992) for fungi and plants. Two regions were amplified: the internal transcribed spacer (ITS) of ribosomal DNA, and the nuclear coding region of elongation factor 1- α (EF-1 α).

Polymerase chain reaction (PCR) was performed using a standard mixture containing 2.5 μ L of 10× buffer, 0.5 μ L dNTPs, 0.5 μ L forward primer (F), 0.5 μ L reverse primer (R), 0.25 μ L Taq polymerase, and nuclease-free water to a final volume of 25 μ L. The primers used were ITS1 and ITS4 for ITS (White et al. 1990), and EF1-728F and EF1-986R for EF-1 α (O'Donnell et al. 1998; Rehner and Buckley 2005). Finally, sample sequencing was performed at the Laboratorio de Servicios Genómicos (LabSerGen) of the Unidad de Genómica Avanzada (UGA) at CINVESTAV Irapuato using capillary (Sanger) sequencing.

Specimens were deposited in the Instituto Tecnológico de Ciudad Victoria. All registered specimen numbers and strain numbers are cited in Table 1.

Phylogeny

The phylogenetic analysis included 58 sequences from the ITS and EF1 regions: 52 were retrieved from NCBI, and 6 were obtained from specimens collected in this research. The sequence alignments and phylogenetic trees generated in this study have been deposited in the Zenodo open-access repository under the DOI 10.5281/zenodo.20318136. They were concatenated using MESQUITE 4.02 (Maddison and Maddison 2025), for a total of 1387 bp. Sequences of *A. phalloides* and *A. verna* were used as the outgroup.

The ML tree was constructed using the HKY+F+I+R2 evolutionary model with 100,000 bootstrap replicates (BS) in IQ-TREE (Nguyen et al. 2015). The BI tree was constructed using the evolutionary model GTR+I+ Γ with 10,000,000 replicates in MrBayes 3.2.7a (Ronquist et al. 2012). Effective sample sizes (ESSs) and data convergence were assessed using Tracer v1.7.2 (Rambaut et al. 2018). The phylogeny was represented using an ML tree; editing was performed in FigTree (Rambaut 2018) and Chiplot (Xie et al. 2023), with values >75% BS and > 0.9 posterior probability (pp) considered significant.

Table 1. Species name, loci (ITS and EF1), and their countries used in this research. ^aThe term "Code" refers to the concatenated sequences within the phylogram.

Species	ITS	EF1	Code	Location
<i>A. phalloides</i>	PP911738.1	JX998000.1	PJ3800	China
	EU909444.1	EU886739.1	EE4439	France
<i>A. virosa</i>	EF493032.1	MH509167.1	EM3267	China
<i>A. rubescens</i>	EU819464.1			USA
<i>A. flavorubens</i>	PX139338.1			USA
<i>A. fritillaria</i>	PQ164613.1	MH508821.1	PM1321	China
<i>A. detera</i>	MN647013.1	MN657466.1	MM1366	China
<i>A. citrinoindusiata</i>	MH508324.1	MH508748.1	MM2448	China
	MH508325.1	MH508747.1	MM2547	China
<i>A. spissacea</i>	PV715482.1	MH509112.1	PM8212	China
	PX688677.1			Viet Nam
	KY940266.1	MH509111.1	KM6611	China
<i>A. citrinoannulata</i>	PX833503.1	MH508743.1	PM0343	China
<i>A. spissa</i>	PV265303.1	PV342182.1	PP0382	China
<i>A. flavosquamosa</i>	ON059324.1	MH508796.1	OM2496	China
<i>A. flavipes</i>	FJ375327.1	PV342172.1	FP2772	China
<i>A. augusta</i>	OR488563.1	MH508678.1	OM6378	Canada
<i>A. fuscusquamosa</i>	MH508373.1	MH508828.1	MM7328	New Zealand
<i>A. sepiacea</i>	PX688741.1	MW324505.1	PM4105	Viet Nam
<i>A. 'ostendemihi'</i>	MZ668039.1			USA
	MK071695.1			USA
<i>A. sinocitrina</i>	PQ858241.1	MN657474.1	PM4174	USA
	LC831430.1			Japan
<i>A. lavendula</i>	PX139565.1			USA

	PX139448.1			USA
<i>A. citrina</i>	KT008018.1	KT008014.1	KK1814	Colombia
<i>A. porphyria</i>	PX411744.1	MH508988.1	PM4488	China
	MN257618.1	MN267405.1	MM1805	Italy
	MH508507.1	MH508989.1	MM0789	Germany
<i>A. citrina_var_intermedia</i>	OW332133.1			Czechoslovakia
<i>A. intermedia</i>	MN257616.1	MN267403.1	MM1603	Italy
<i>A. citrina</i>	PV938811.1	MH508733.1	PM1133	Hongkong
<i>A. 'americitrina'</i>	MT511607.1			USA
	MT511606.1			USA
<i>A. brunneolimbata</i>	PV564115.1	MH508697.1	PM1597	China
<i>A. elongata</i>	PP796189.1	MH508766.1	PM8966	Canada
<i>A. brunneolocularis</i>	OR506128.1	OR498115.1	OO2815	México
<i>A. orsonii</i>	PV962332.1	MN657465.1	PM3265	China
<i>A. rubescens</i>	PV265288.1	MW874595.1	PM8895	China
	MK492597.1	PX363637.1	MP9737	Spain
<i>A. brunneolimbata</i>	MH508277.1	MH508696.1	MM7796	China
	MH508276.1			China
<i>A. brunnescens</i>	OR987278.1	AY881021.1	OA7821	USA
	MH211999.1			USA
	PX139354.1			USA
	PZ387659	PZ405185	ITCV 1913	México
	PZ387660	PZ405186	ITCV 1914	México
	PZ387661	PZ405187	ITCV 1915	México
	PZ387662	PZ405188	ITCV 1916	México
	PZ387663	PZ405189	ITCV 1917	México
	PZ387664	PZ405184	ITCV 1918	México
	PV748894.1			USA
	MH212122.1			USA
<i>A. parvifritillaria</i>	OR345189.1	MN657472.1	OM8972	China
	ON768714.1	MN657471.1	OM1471	China
<i>A. fritillaria</i>	PQ858243.1	MH508820.1	PM4320	China
	FJ375328.1	ON325626.1	FO2826	China
	FJ441031.1	MN657470.1	FM3170	China

Species delimitation

Species delimitation used the Assemble Species by Automatic Partitioning (ASAP) method (Puillandre et al. 2021) with the Kimura 2-parameter (K80) evolutionary model for the ITS and EF1 alignments, and the General Mixed Yule Coalescent (GMYC) (Fujisawa and Barraclough 2013) for the concatenated alignment. The GMYC analysis required constructing an ultrametric tree in BEAST v1.10.5, using BEAUti (Drummond et al. 2012) to configure the input files for the R platform v4.5.2 (R Core Team 2025), where the tree was obtained, and finally a Neighbor-joining analysis in MEGA (Kumar et al. 2024).

Results

Taxonomy

Amanita brunnescens (G. F. Atk.). Figs. 1 A-F. Type: found in oak forest in association with madroño and pinguica, Mexico: Guanajuato, Cuenca de la Esperanza, Las Palomas, ITCV 1918. Gene sequences: PZ387659 (ITS), PZ405185 (EF-1 α).

Basidioma: Small, solitary growth; Pileus: 34 × 45 mm convex, nearly uniform color, light yellowish brown, darker at the apex; partial universal veil as small cottony patches. Surface dry, waxy; margin incurved, entire, striate, not appendiculate; context uniformly white. Lamellae: white to cream, complete lamellae, free, crowded, with smooth margin.

Stipe: 16 × 26 mm, clavate bulbous, uniformly light whitish. Surface finely striated parallel to the stipe; context white, solid. Annulus: central, thick, membranous, and friable at the edges, finely striated on its surface. Universal veil: white, partial or absent only at the edge of the volva. Volva: circumcissile, white, without remnants on the stipe.

Microscopic characters: trama divergent. Mediostratum: 30–50 μ m thick, composed of hyphae 6–12 μ m thick with septa 6–12 μ m. Lateral strata: 30–50 μ m. Hymenium: 40–60 μ m thick, with 1–2 layers of square, rhomboid to prismatic or irregular cells. Basidia: (–)9–13(–15) × (7–)8–11(–12) μ m, clavate, tetrasporic; sterigmata 2–6 μ m long. Basidiospores: (7–)8–12(–13) × (6–)7–10(–11) μ m, Q = 1.00 × 1.43 (–1.50), Q = 1.19, mostly globose to slightly ellipsoid, colorless, hyaline in 5% KOH, thin-walled, smooth. Suprapellis: 120–200 μ m, composed of interwoven hyphae, hyphae 5–8 μ m thick; subpellis: 100–150 μ m of prostrate hyphae, hyphae 5–10 μ m thick, filamentous to thin, septa 2–8 μ m, hyaline in 5% KOH; hyaline, without vacuolar content, thin-walled. Volva: composed of a layer 120–180 μ m thick, hyphae 5–10 μ m, septa 5–8 μ m, and sphaerocytes (9–)10–36 × (7–)8–27 μ m.

Molecular phylogeny

According to the ASAP delimitation program, using the concatenated alignment and ITS and EF1 alignments separately, together with sequences from the U.S. and Canada, the sequences showed different groupings: the ITS section grouped the collections into a single group, except for ITCV 1916 and ITCV 1917. The EF1 region grouped the collections with the concatenated sequence OA7821 of *A. brunnescens* from the U.S. Meanwhile, GMYC, using the concatenated alignment, grouped ITCV 1916 with the rest of the U.S. sequences and separated the remaining sequences from this study into their own category (Fig. 2).

The ML and BI trees obtained from concatenating the ITS and EF1 regions were very similar. Within these, the species of interest were grouped into a clade formed by *A. brunnescens* specimens from the eastern United States (U.S.), with bootstrap support >75% and posterior probability > 0.5. This result establishes the relationship between the species from central Mexico and those sequenced from the eastern U.S.

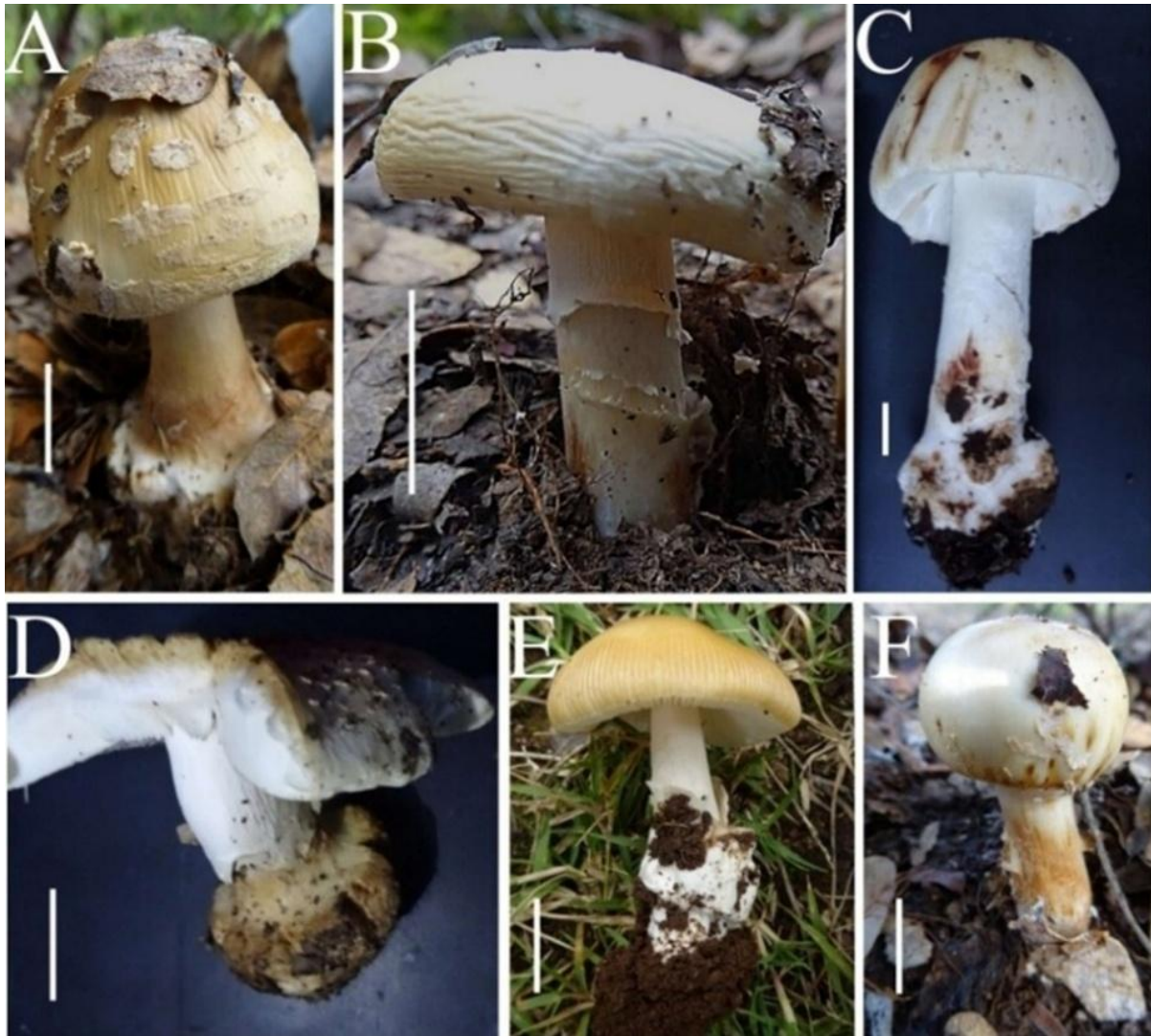


Figure 1. A–F; Collections made in the state of Guanajuato, showing morphological variation among the collections, where we can see the absence of the annulus (A, D, E, F) or absence of veil remnants on the volva (A, C, F). Bars: A–F = 2 cm.

Discussion

Amanita brunnescens is a species with a distribution in Mexico; however, its presence has not been reported in Guanajuato. In the protected natural area 'Cuenca de la Esperanza' (specifically at the “Las Palomas” station), the vegetation is dominated by oak forest (*Quercus* spp.) in association with *Arbutus* spp. and *Arctostaphylos pungens* (Padilla 2010). This habitat setting directly aligns with the documented ectomycorrhizal association of *A. brunnescens* with *Quercus* hosts across its North American distribution (Jenkins 1986; Tulloss et al. 1995; Aroche et al. 1982). Furthermore, our results suggest a relationship of the group of interest with North American species, as well as some diversity within the group of interest itself.

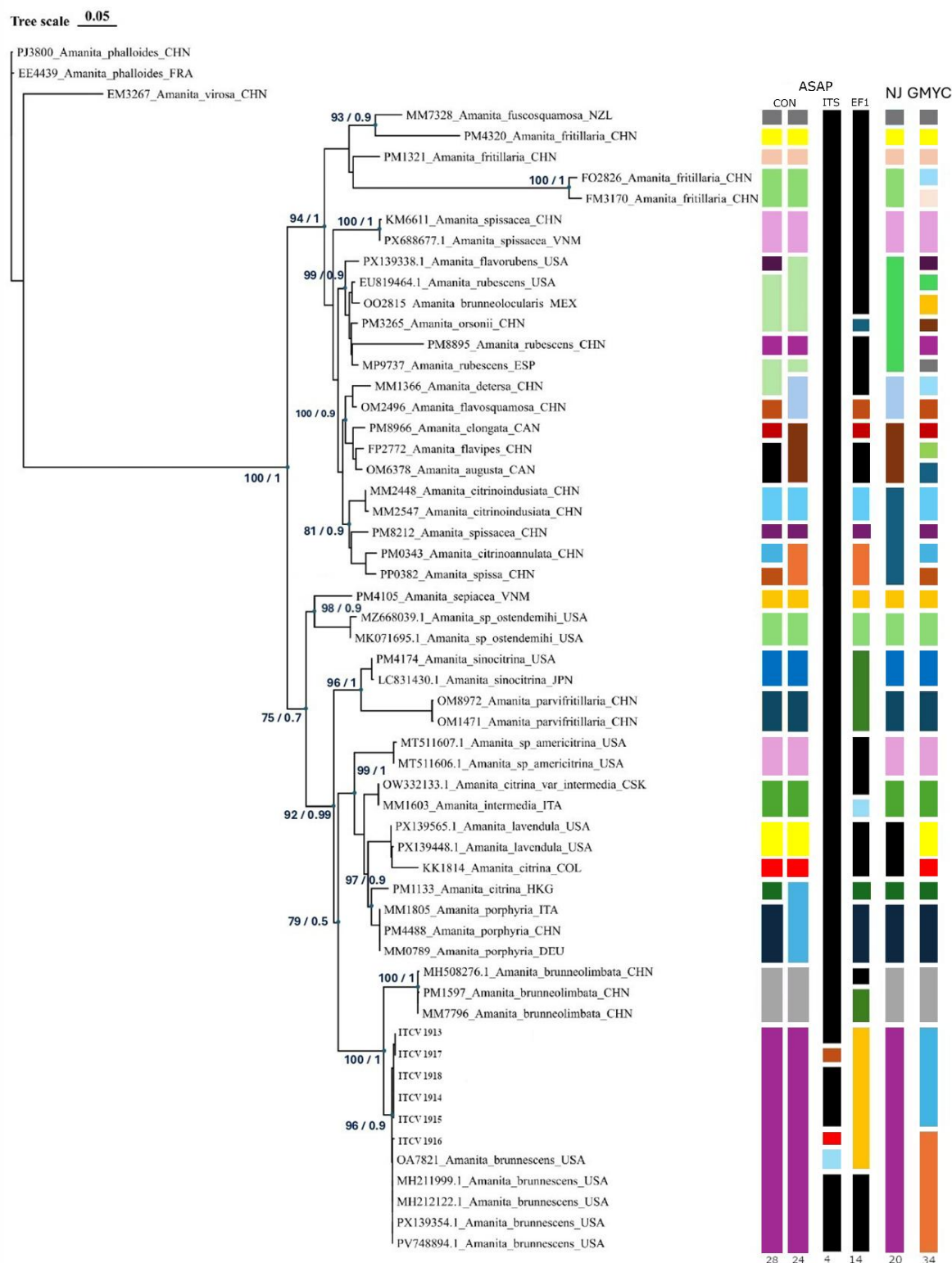


Figure. 2. Maximum likelihood phylogram of concatenated ITS and EF1 sequences with support values >75% BS and >0.5 pp, showing the results obtained by Neighbor joining, ASAP (for ITS and EF1), and GMYC.

Morphology

Collections ITCV 1913 and ITCV 1917 exhibit a pale cream coloration, very similar to *Amanita brunnescens* var. *pallida* Louis Charles Christopher Krieger (1927). Even so, ITCV 1913 has a broad, membranous apical annulus, whereas ITCV 1917 is a young specimen with no annulus observed.

The remaining collections share a light-brown surface coloration resembling *A. brunnescens*, although they differ in several respects. The veil of the volva is only present in ITCV 1915. Likewise, inconsistencies are observed in the pileus striation: ITCV 1915 is broadly striated, sulcate with a crispate margin, whereas collections ITCV 1918 and ITCV 1914 lack striations. Finally, the stipe is mostly finely striated in all collections except for ITCV 1918. Taking these differences into account, it is possible to relate the groups to the typical morphologies of *A. brunnescens* and their pale variety, albeit with some differences.

The comparison of the collections made in this study with those described for the taxon suggests that the species shares similarities, such as an abruptly bulbous body, floccose warts on the surface, a striate margin in mature specimens, and coloration ranging from brown to pale. However, important differences are found in the presence or absence of structures such as the annulus, the volva, or the pileus surface, which lead us to think that these could be varieties within the species, perhaps due to geographic location or soil composition.

Species delimitation and marker evaluation

In the results obtained, we can see a well-supported clade (96 bootstrap and 0.9 posterior probability) with a tendency towards grouping between the sequences from this study and those from the U.S., or towards belonging to a single group, although with relevant discrepancies depending on the marker and the method used.

ASAP, in its two analyses with concatenated data, concluded a relationship with the North American sequences, indicating a marked phylogenetic affinity. However, when performing the analysis with the separate regions, it was established that, at least for ITS, it distinguishes ITCV 1916 and ITCV 1917 as unique sequences, without grouping them, possibly due to the variability of this region in these lineages. In addition, the EF1 analysis showed congruence with the conservation or affinity with at least one North American sequence, possibly due to the conserved nature of this gene.

In the GMYC analyses, all sequences of interest were grouped except ITCV 1916, which was grouped with the North American sequences. This may suggest early differentiation or genetic structure within the group of interest. Finally, the NJ tree grouped all the sequences obtained in Guanajuato with the North American sequences, supporting the earlier observation in some results that these belong to the same species or clade.

The disparity in this data may be due to factors such as the evolutionary rate of the markers used or the limitations of the analytical methods employed. Furthermore, the study could be further strengthened by incorporating additional genetic regions (e.g., LSU, β -tubulin, and RPB2) to achieve more conclusive results. Nevertheless, despite these limitations, the results may indicate genetic diversity within

the group of interest, a single lineage present in the north and center of the continent, and a possible incipient point of speciation for the *brunnescens* taxon.

Table 2. Comparative table of morphological characteristics of *A. brunnescens*, showing the differences between the presence or absence of defined characters for the species and their comparison with the description by George Francis Atkinson.

	Pileus	Veil remnants	Stipe	Volva	Spores
<i>Amanita brunnescens</i> (G. F. Atk.) E.-J. Gilbert. 1940	Smoky brown surface, darker in the center, frequently streaked or spotted with brown, sometimes slightly striate at maturity.	As flocculose scales, white to pale ochre.	Flocculose-squamulose, broad apical annulus, membranous, white to mouse gray, abruptly bulbous, brown when handled.	Veil remnants on the bulb margin.	(7-) 8 – 9 (–9.5) × (6.5-) 7 – 8.5 (–9)
ITCV 1913	Whitish cream surface, smooth, pale to cream context, with irregular longitudinal striations of variable depth, with color change to brown, incurved and striate margin.	Almost absent, scattered, and cottony in appearance, white or light brown.	Snakeskin-shaped texture, apical annulus, membranous and finely striate, abruptly bulbous, light brown in damaged areas.	Circumscissile, with friable remnants on the edge and flocculose at the base.	(8-) 9 – 11 × (6-) 7 – 9
ITCV 1914	Dark brown surface at the apex and lighter, almost white toward the margin; whitish cream and light brown context towards the margin; margin straight and striate.	As small patches with uniform distribution, light brown or cream.	Finely striate, without annulus or veil remnants at the base, abruptly bulbous, cream-white and dark brown at the base.	Almost absent, with slight brown to gray remnants on the edge.	(7-) 8 – 13 (–15) × 6 – 10 (–11)
ITCV 1915	Pileus concolorous, light brown in surface and context; surface smooth and shiny; margin straight and strongly striate at the edge.	None on the surface.	Finely striate, without annulus or veil remnants on the surface, bulbous body white to cream.	Sacciform, white, with a broad veil.	(7-) 8 – 12 (–13) × (6-) 7 – 10 (–11)

ITCV 1916	Surface dark brown to light brown; margin with straight horizontal striations over the edge.	Cottony, patches.	as	Finely striate, central annulus and without veil remnants on its surface, bulbous body, light to dark brown.	Without volva.	9 – 13 (–15) × (7–) 8 – 11 (–12)
ITCV 1918	Pileus concolorous, dark brown to light brown in surface and context; margin straight with a slightly radially wrinkled surface.	With uniform distribution, as cottony patches, cream-white to brown.		Snakeskin-shaped texture, without annulus or veil remnants, brown to dark when handled, abruptly bulbous.	Without volva.	8 – 14 (–15) × (6–) 7 – 11 (–12)

Conclusions

Phylogenetic analyses place the specimens from Guanajuato directly within the North American *Amanita brunnescens* clade. Despite clear morphological differences, these sequences remain highly conserved. This genetic link is further reinforced by their shared host specificity, as both groups associate with *Quercus* species across North America. The observed phenotypic variation likely stems from local soil conditions, microclimates, and regional adaptations.

Disclosure

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the country where they were performed.

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