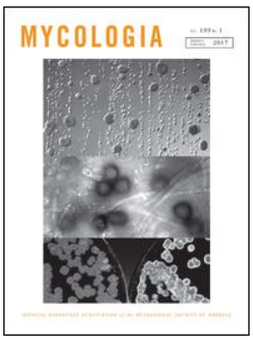

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Defining the phylogenetic position of *Amanita* species from Andean Colombia

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ABSTRACT

Amanita is a worldwide-distributed fungal genus, with approximately 600 known species. Most species within the genus are ectomycorrhizal (ECM), with some saprotrophic representatives. In this study, we constructed the first comprehensive phylogeny including ECM species from Colombia collected in native *Quercus humboldtii* forests and in introduced *Pinus patula* plantations. We included 8 species (*A. brunneolocularis, A. colombiana, A. flavoconia, A. fuligineodisca, A. muscaria, A. rubescens, A. sororcula*, and *A. xylinivolva*) out of 16 species reported for the country, two new reports: *A. citrina* and *A. virosa*, and a new variety *A. brunneolocularis* var. *pallida*. Morphological taxonomic keys together with a phylogenetic approach using three nuclear gene regions: partial nuc rDNA 28S nuc rDNA internal transcribed spacers ITS1 and ITS2 and partial translation elongation factor 1-α gene (*TEF1*), were used to classify the specimens. Several highly supported clades were obtained from the phylogenetic hypotheses obtained by Bayesian inference and maximum likelihood approaches, allowing us to position the Colombian collections in a coherent infrageneric level and to contribute to the knowledge of local *Amanita* diversity.

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Acrophysalids; *Amanita*; infrageneric classification; molecular systematics; rDNA; translation elongation factor 1-α gene

INTRODUCTION

Fungi in the genus *Amanita* Pers.: Hooker are morphologically characterized by producing a white to cream spore print, gills that are free to slightly adnexed to the stipe, and the presence of morphologically diverse universal veils. Microscopic characteristics include a divergent hymenophoral trama and smooth amyloid or inamyloid spores. In the past, several classification systems have been proposed for this genus, on the basis of a combination of morphological characteristics. Corner and Bas (1962) and Bas (1969) initially separated the species of *Amanita* into two widely accepted subgenera: those belonging to the subgenus *Amanita*, characterized by a striate to plicate pileus margin and inamyloid spores, and those belonging to the subgenus *Lepidella*, characterized by an even pileus margin and amyloid spores. More precisely, according to Bas (1969), the variation in the universal veil traits and whether or not the pileus margin has an appendiculate aspect were used to classify species into six sections: *Amanita* and *Vaginatae* in the subgenus *Amanita* and *Amidella, Lepidella, Phalloideae*, and *Validae* in the subgenus *Lepidella*. On the basis of this primary classification, Singer (1986) added four sections: *Caesareae, Mappae, Ovigerae*, and *Roanokenses*, to separate species according to specific morphological differences, such as the presence or absence of an annulus, subtle variations in the volva, as well as microscopic features.

Over the last 15 years, different rearrangements of infrageneric sections for the genus have been hypothesized on the basis of molecular phylogenetic analyses of nuc rDNA sequences, following the traditional classification systems (Bas 1969; Singer 1986). Although no clear split of the genus into the major subgenera *Amanita* and *Lepidella* (Bas 1969) has been highly supported in most of the molecular studies, Weiss et al. (1998) confirmed the monophyly of most of the sections. Later, Oda et al. (1999) proposed a subdivision of three sections within the subgenus *Amanita* and a modification of the taxonomic treatments at the section level within the subgenus *Lepidella*. Drehmel et al. (1999) proposed a classification using partial 28S rDNA (28S) sequences, providing a rearrangement into seven subsections (*Amanita, Amidella, Caesareae, Ovigerae, Phalloideae, Vaginatae*, and *Validae*) and two series (*Mappae* and *Validae*). A study by Zhang et al. (2004) established the relationships among eastern Asian species on the basis of a phylogenetic hypothesis of nuc rDNA ITS1-5.8S-ITS2 (internal transcribed

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spacer [ITS]) and partial 28S sequences. They obtained highly supported clades corresponding to sections *Amidella* and *Validae* when analyzed by distance-based methods (neighbor joining), and to sections *Amanita, Vaginatae*, and *Validae* when using maximum parsimony analysis; however, the initial division of the genus into two subgenera, *Lepidella* and *Amanita*, was not strongly supported in any of their analyses. More recently, a number of studies have included asymbiotic species, with some contrasting taxonomic arrangements. Vizzini et al. (2012) recovered the asymbiotic species as a clade, sister to the genus *Lepidella*, and proposed splitting the genus into two genera, *Amanita* and *Aspidella*. Wolfe et al. (2012) proposed a multigene phylogenetic hypothesis for the genus, which showed a monophyletic *Amanita*, with ectomycorrhizal (ECM) and saprothrophic species as two reciprocally monophyletic clades. Following this phylogenetic reconstruction, Redhead et al. (2016) proposed to split the genus into two genera and place the ectomycorrhizal species within the genus *Amanita* and the saprotrophic species within a new genus, *Saproamanita*, in addition to new infrageneric levels. More recently, Tulloss et al. (2016) challenged this proposal, arguing that the genus *Amanita* should not be split due to the lack of support and monophyly of the saprotrophic species group, limited taxon sampling, lack of phylogenetic evidence-based on a low number of genes, among others, and recommended keeping both saprotrophic and ECM species within the single genus *Amanita*.

To date, a total of 16 species of *Amanita* have been reported in Colombia (Singer 1963; Nasi 1977; Pulido 1983; Tulloss et al. 1992; Franco-Molano and Uribe-Calle 2000; Franco-Molano et al. 2000; Halling and Mueller 2005; Vasco-Palacios and Franco-Molano 2013). Singer (1963) reported *Amanita inaurata* Secr. and *A. humboldtii* Sing; *A. rubescens* was reported initially by Nasi (1977) and *A. muscaria* was initially reported by Pulido (1983); new species were described by Tulloss et al. (1992): *A. advena, A. arocheae, A. aureomonile, A. brunneolocularis, A. colombiana, A. fuligineodisca, A. picea, A. sororcula*, and *A. xylinivolva; A. gemmata* was initially reported by Guzman and Varela (1978); *A. flavoconia* by Saldarriaga et al. (1988), Tulloss et al. (1992), Franco-Molano et al. (2000), Franco-Molano and Uribe-Calle (2000), Halling and Mueller (2005), and Vasco-Palacio and Franco-Molano (2013); and *A. savannae* by Tulloss and Franco-Molano (2008). In total, 14 taxa represent the sections *Amanita, Phalloideae*, and *Vaginatae*; the species *Amanita advena* represents the section *Lepidella*; and *A. savannae* represents the subsection *Vittadiniae*, within the saprotrophic species group (Tulloss and Franco-Molano 2008). Most of these species are reported to be associated with *Quercus humboldtii*, a tree species endemic to northern South America and in the Darién region of Panamá (Orwa et al. 2009), and distributed in the three cordilleras of Colombia over a range of altitudes from 750 to 3450 m above sea level (masl; Fundación Natura 2007); on the contrary, *A. muscaria* and *A. rubescens* are commonly associated with *Pinus* spp. introduced to Colombia. No species from section *Caesareae* have been found in Colombia; these species are mainly distributed in temperate regions and other tropical regions different from the South American neotropics (Sánchez-Ramirez et al. 2015).

The Andean montane forests support a high biodiversity of organisms (Bush et al. 2011), and it is estimated that the diversity of fungi in oak forests is high (Franco-Molano et al. 2000). Although a high number of Agaricales in Colombia have been described according to morphological traits (Halling and Ovrebo 1987; Halling 1989a, 1989b; Horak and Halling 1991; Singer et al. 1995; Franco-Molano 1999), the phylogenetic relationships of species in this order have not been explored yet in Colombia. In this study, specimens representing 10 species of *Amanita* collected in Colombia were identified from morphological characteristics and positioned in a global phylogenetic context by maximum likelihood (ML) and Bayesian inference (BI) approaches, allowing infrageneric-level classification. On the basis of the combined results of the morphological analysis and the position of the Colombian samples in the phylogeny, some taxonomic topics on the infrageneric ranks of the genus are discussed, a new variety is proposed, and two new species records are reported.

MATERIALS AND METHODS

Fungal collection.—Seventy-seven basidiomes of the genus *Amanita* were collected between Mar 2012 and Jun 2014 in the northeastern cordillera of the Colombian Andes: Departamento de Boyacá, Municipio de Villa de Leyva, Vereda Capilla, 5°39′26.78″N, 73°30′46.41″W; Departamento de Boyacá, Municipio de Arcabuco, Vereda Piedras Blancas, 5°48.546″N, 73°28.751″W; Departamento de Boyacá, Municipio de Arcabuco, 5°45′ 35.38″N, 73°26′47.10″W; and Departamento de Santander, Municipio de Belén, vereda San José de La Montaña, 6°2′ 29.82″N, 72°59′99.8″W. The herbarium collection *A. fuligineodisca* AFM1812 collected in Departamento de Antioquia, Corregimiento de Santa Elena, Estación biológica Piedras Blancas, stored in HUA (Herbario de la Universidad de Antioquia, Medellín, Colombia), was included in the molecular analysis. The fungi were collected in *Quercus humboldtii* and in *Pinus patula* forests. Macroscopic features were described; color designations were assessed according to Kornerup and Wansher (1978) (given within parentheses); microscopic

measurements were made in samples treated with 3% KOH or Melzer's reagent. The notation for basidiospore measurements are as follows: [a/b/c] where a is the number of basidiospores measured from b basidiomes taken from c collections. Lengths (L) and widths (W) of 30 spores and 30 basidia were measured, and the Q' value (average ratio of length/width for all spores measured for all specimens of a species) was calculated (Tulloss 2005b). Macroscopic tests with 3% KOH were carried out on the pileus and stipe tissues. Once specimens were described, they were dried, packaged in plastic bags, registered in the SPECIFY database, and stored at the ANDES Herbarium (Universidad de los Andes, Bogotá, Colombia). Based on macro- and microscopic characters, specimens were assigned to species according to Bas (1969), Jenkins (1986), Singer (1986), Tulloss et al. (1992), Franco-Molano et al. (2000), Tulloss (2002, 2005a, 2008, 2009a, 2009b), Halling and Mueller (2005), and Tulloss and Possiel (2005).

DNA extraction, amplification, and sequencing.—DNA was obtained from 24 dried basidiomes by the protocol reported by Zolan and Pukkila (1986), with the following modifications: the lysis buffer consisted of 2.5% cetyl trimethyl ammonium bromide (CTAB), 100 mM Tris, pH 8, 20 mM ethylenediaminetetraacetic acid (EDTA), 1.4 M NaCl, 1% polyvinyl pyrrolidone (PVPP) 40 000, and 1% PVPP 360 000 (Zolan and Pukkila 1986).

Primers ITS4 and ITS5 (White et al. 1990) were used to amplify the ITS region. PCR was performed with a Peltier thermal cycler (Bio-Rad, Foster City, California, USA) in 25-µL reaction mixtures containing double-distilled H_2O, 1 µL of DNA template, 0.5 µL of each 10 µM primer, 2.5 µL of Taq 10× buffer, 0.5 µL of 10 mM dNTP mix, 2 µL of 25 mM $MgCl_2$, and 1 µL of 5 U/µL *Taq* polymerase. Cycling parameters were as follows: initial denaturation at 94 C for 1 min, followed by 35 cycles of denaturation at 96 C for 2 min, annealing at 55 C for 1 min, and extension at 72 C for 2 min, and a final extension at 72 C for 10 min. Polymerase chain reaction (PCR) amplification of the first 700 bases corresponding to the D1–D2 region in the 28S gene was carried out with primers LR0R and LR7 (Vilgalys and Hester 1990; Rehner and Samuels 1994), under the reaction conditions mentioned above with the following thermal cycling parameters: initial denaturation at 94 C for 4 min, followed by 35 cycles of denaturation at 94 C for 30 s, annealing at 54.5 C for 1 min, and extension at 72 C for 1 min, and a final extension at 72 C for 5 min. In addition to the nuclear ribosomal regions, which have proven to be informative in reconstructing the phylogenetic relationships within *Amanita* and other closely related taxa (Drehmel et al. 1999; Zhang et al. 2004; Justo et al. 2010) and are commonly used as phylogenetic fungal barcodes (Schoch et al. 2012), we used the translation elongation factor 1-α (*TEF1*) protein-coding gene, which is informative in low-level phylogenetics approaches (Rehner 2001). PCR amplification of *TEF1* gene was carried out with the primers 983F and 1567R under the PCR conditions given by Rehner and Buckley (2005). Amplified PCR products were visualized by gel electrophoresis on a 1% agarose gel. PCR products were sequenced with a 3730xl DNA analyzer (PE Applied Biosystems, Carlsbad, California, USA). Forward and reverse sequences were assembled with Geneious Basic 4.8.5 (Biomatters Ltd., Auckland, New Zealand) and using BLAST, compared against the nucleotide bases of GenBank and UNITE (Kõljalg et al. 2013; http://unite.ut.ee/cite.php) to confirm that the sequence obtained corresponded to the target organism.

Phylogenetic analyses and species delimitation.—A total of 220 ITS, 156 28S, and 39 *TEF1* gene sequences from ectomycorrhizal (ECM) *Amanita* species (SUPPLEMENTARY TABLE 1) were used to construct the consensus phylogeny. The sequences retrieved from GenBank that had voucher collections were used in the analysis, whereas those corresponding to soil samples were not included. A data set containing the concatenated alignments of the ITS, 28S, and *TEF1* alignment was generated. The incongruence length difference (ILD; Farris et al. 1994) test was used to analyze for instances of incongruence among the used markers. This data set consisted of a matrix that resembles the phylogenetic approach presented by Sanderson et al. (1998), as there were missing data. An independent phylogenetic tree for each gene was constructed by Bayesian inference (SUPPLEMENTARY FIGS. 1–3). The alignments were performed with MAFFT (Katoh et al. 2002) and curated by Gblocks (Castresana 2000) with default parameters. Sequence statistics of each gene and concatenated alignments were calculated with MEGA 5 (Tamura et al. 2011). The alignment file can be accessed on TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S20976). Bayesian inference was performed with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). Fifty million Metropolis-coupled Markov chain Monte Carlo (MCMCMC) generations were run with a sample frequency of 1000 and a burn-in of 10% of the total, specifying a different model of substitution per gene with jModelTest (Posada 2008). Two runs using four chains each, one cold and three heated chains, were performed, and the results of each chain were summarized to obtain the majority consensus trees from the total run, collapsing all branches with posterior values less than 0.5

(Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Each run was examined with Tracer v1.6.0 (Rambaut and Drummond 2013) to determine whether the burn-in procedures were correctly assumed and whether there was convergence between the chains and the runs. A maximum likelihood (ML) analysis was performed by RAxML (Stamatakis et al. 2008) with the GTRCAT substitution model with 1000 bootstrap iterations to assess clade support. We used *Pluteus romelli* and *Limacella glioderma* as outgroups. Phylogenetic species and monophyletic group recognition was performed by the identification of highly supported clades on the phylogeny (i.e., ML >75% bootstrap support [BS] or >0.95 Bayesian posterior probability [PP]) sensu Dettman et al. (2003).

RESULTS

A total of 77 basidiomes were collected and classified in 10 species, among which 24 specimens were included in the phylogeny of the genus (TABLE 1). A total of 415 sequences from the three genes, corresponding to 106 *Amanita* species, were used to construct the phylogenetic trees, (SUPPLEMENTARY TABLE 1). The ITS, 28S, *TEF1*, and the combined data sets consisted of 1117, 662, 575, and 2356 characters (including gaps); 697, 217, 211, and 1125 parsimony-informative characters; 863, 310, 245, and 1418 variable sites; and 195, 328, 325, and 848 conserved sites, respectively. The ILD test detected no significant (P = 0.01) incongruence among the markers used.

Both ML- and Bayesian-based reconstruction hypotheses yielded similar phylogenies, grouping five species (*A. colombiana* Tulloss, Ovrebo & Halling, *A. fuligineodisca* Tulloss, Ovrebo & Halling, *A. muscaria* (L.), *A. sororcula* Tulloss, Ovrebo & Halling, and *A. xylinivolva* Tulloss, Ovrebo & Halling) within the subgenus *Amanita* and five species (*A. citrina* Pers., *A. flavoconia* G.F. Atk., *A. virosa* Bertill., *A. rubescens* Pers., and *A. brunneolocularis* var. *pallida*, var. nov) in the subgenus *Lepidella*. FIG. 1 shows some of the species' basidiomes, spanning the sections *Amanita, Phalloideae*, and *Vaginatae*.

Subgenus *Amanita*.—An initial morphological classification of 49 specimens in the subgenus *Amanita* was determined with taxonomic keys (Jenkins 1986; Tulloss et al. 1992; Franco-Molano et al. 2000; Tulloss 2000, 2008; Halling and Mueller 2005). Of these, 20 specimens were classified in the section *Vaginatae* (TABLE 1), all of them without an annulus: 17 specimens determined as *A. fuligineodisca* (FIG. 1A), 3 as *A. colombiana* (FIG. 1B), and 1 as *A. sororcula* (FIG. 1C).

The division of the subgenus *Amanita* into two sections, *Amanita* and *Vaginatae*, accepted by the traditional classifications was supported by the Bayesian analysis (FIG. 2) and is further supported by several morphological characters. Species in section *Amanita* are mainly differentiated from those in section *Vaginatae* by having a bulbous stipe bases and nonsaccate volva (Jenkins 1986; Singer 1986; Drehmel et al. 1999). According to the concatenated phylogeny within subsection *Vaginatae* sensu Drehmel et al. (1999), two clades were separated, corresponding to the series *Ceciliae* (FIG. 2, clade d) and series *Fulvae* (FIG. 2, clade e) proposed by Tulloss and Yang (2012a, 2012b). *Amanita colombiana* (FIG. 1B), a species characterized by its red volva, and *A. sororcula* (FIG. 1C), characterized by its submembranous, pale gray volva, grouped within the series *Ceciliae* (FIG. 2, clade d). *Amanita fuligineodisca* clearly clustered together with *A. fulva* Fr., in stirps *Fulva* (Tulloss and Yang 2012c) (FIG. 2, clade h).

Twenty-nine specimens were determined in the section *Amanita*, grouping 17 in the species *A. muscaria* and 12 in the species *A. xylinivolva* (TABLE 1). The basidiomes were mainly diagnosed by differences in the pileus and volva. Basidiomes classified as *A. xylinivolva* (FIG. 1D) were characterized by pale yellow to pale gray pileus coloration, a pileus surface with nonuniform volval remnants, a thin membranous ring that disappears in mature specimens, a limbate volva, and inamyloid globose to subglobose spores. Following Tulloss (2009a), *A. farinosa* differs from this species by a brownish gray pileus and a pulverulent volva, among others. According to the phylogeny, these samples are closely related to the species *A. stranella*, characterized by a citron or straw- to cream-colored pileus, membranous ring, and volva with a marginal limb (Tulloss and Yang 2015a). Moreover, *A. xylinivolva* grouped in a different clade from that of the similar North American *A. gemmata*. The latter species has been reported by some authors from Colombia (Nasi 1977; Pulido 1983; Henao and Tobon 1984). On the other hand, basidiomes of *A. muscaria* were characterized by a polished pileus surface that was red to orange, or sometimes blood-red, with white to yellowish cream patches of volva on the pileus surface, and a volva formed by superficial membranous or scaly warts at the base of stipe.

Subgenus *Lepidella*.—The division of the subgenus *Lepidella* into two sections *Lepidella* and *Phalloideae* was supported in the phylogeny by the BI analysis (FIG. 2). This division is supported morphologically by the

Table 1. Rank categories below section level of the collected species according to four classification systems.

Species	Collected sporocarps /Collections /Samples in the phylogeny /Host /Distribution*	Classification according to			
		Drehmel et al. (1999)	Taxonomic keys Jenkins (1986) and Tulloss**	Amanitaceae.org	This Study
A.	*brunneolocularis* var. *pallida*	3 /NVE698, NVE624, IP24 /2 /*Q. humboldtii* /**BOY**	Sect. *Phalloideae*, Subsect. *Validae*, Series *Validae*	Sect. *Validae*	Sect. *Validae*
Sect. *Phalloideae*, Subsect. *Validae*, Series *Validae*, clade k (FIG. 2)					
A. citrina	2 /NVE600, 616 /2 /*Q. humboldtii* /**BOY**	Sect. *Phalloideae*, Subsect. *Validae*, Series *Mappae*	Sect. *Validae*	Sect. *Validae*, Series *Mappae*, Stirps *Bulbosa*	Sect. *Phalloideae*, Subsect. *Validae*, Series *Mappae*, Stirps *Bulbosa*
A. colombiana	3 /NVE410, Penagos3, DS7 /3 /*Q. humboldtii* /ANT, **BOY**	Sect. *Vaginatae*	Sect. *Vaginatae*, Series *Ceciliae*, Stirps *Ceciliae*	Sect. *Vaginatae*, Subsect. *Vaginatae*, Series *Ceciliae*, clade d (FIG. 2)	
A. flavoconia	13 /NVE242, 314, 351, 354, 397, 411, 454b, 521, 533, 563, 627, 746, CV3 /3 /*Q. humboldtii* /ANT, **BOY**, CUN, NAR	Sect. *Phalloideae*, Subsect. *Validae*, Series *Validae*	Sect. *Validae*	Sect. *Validae* infrasp. taxa of *flavoconia*	Sect. *Phalloideae*, Subsect. *Validae*, Series *Validae*, clade j (FIG. 2)
A. fuligineodisca	18 /NVE286b, 240, 304, 324, 343, 455, 505, 522, 567, 569, 630, 644, 665b, 692, 743, 748, AFM1812 /2 /*Q. humboldtii* /ANT, BOY, CUN, **SAN**, NAR	Sect. *Vaginatae*, Subsect. *Vaginatae*	Sect. *Vaginatae*	Sect. *Vaginatae*, Series *Fulvae*, Stirps *Fulva*	Sect. *Vaginatae*, Subsect. *Vaginatae*, Series *Fulvae*, Stirps *Fulva*
A. muscaria	17 /NVE277, 157, 488, 500, 588, 590, 636, 649, 662, 664, 674, 680, 719, 725, 726, 750, 760 /3 /*Pinus patula* /ANT, BOY, CAL, CUN, **SAN**	Sect. *Amanita*	Sect. *Amanita*	Sect. *Amanita*, Subsect. *Amanita*, Series *Amanita*, Stirps *Muscaria*	Sect. *Amanita*, clade b (FIG. 2)
A. rubescens	10 /NVE160, 285, 599, 613, 618, 624 /2 /*Pinus* spp. /ANT, CUN, **BOY**	Sect. *Phalloideae*, Subsect. *Validae*, Series *Validae*	Sect. *Validae*	Sect. *Validae* infrasp. taxa of *rubescens*	Sect. *Phalloideae*, Subsect. *Validae*, Series *Validae*, clade k (FIG. 2)
A. sororcula	1 /NVE587 /1 /*Q. humboldtii* /ANT, BOY	Sect. *Vaginatae*, Subsect. *Vaginatae*	Sect. *Vaginatae*	Sect. *Vaginatae*, Series *Ceciliae*, Stirps *Sororcula*	Sect. *Vaginatae*, Subsect. *Vaginatae*, Series *Ceciliae*, clade g (FIG. 2)
A. virosa	3 /NVE74, 410a, 473 /3 /*Q. humboldtii* /ANT, **BOY**, VAL	Sect. *Phalloideae*, Subsect. *Phalloideae*	Sect. *Phalloideae*	Sect. *Phalloideae*	Sect. *Phalloideae*, Subsect. *Phalloideae, virosa* clade (FIG. 2)
A. xylinivolva	12 /NVE56, 126, 490, 491, 504, 511, 535, 670, 671, 672, 735, 747 /3 /*Q. humboldtii* /ANT, **BOY**, CAU, CUN, NAR, **SAN**	Sect. *Amanita*	Sect. *Amanita*	Sect. *Amanita*	Sect. *Amanita*, clade a (FIG. 2)
Total	No. of collected sporocarps = 77; No. of specimens in the phylogeny = 24				

*Departments: Antioquia (ANT), Boyacá (BOY), Caldas (CAL), Cundinamarca (CUN), Nariño (NAR), Santander (SAN), Valle del Cauca (VAL). References: Nassi (1977), Tulloss et al. (1992), Franco-Molano et al. (2000), and Vasco-Palacios and Franco-Molano (2013). In bold are new distributions according to this study.

**References: Tulloss et al. (1992), Tulloss (2002), and Tulloss (2005) or Tulloss (2009a).

Figure 1. Basidiomes of a subset of species analyzed in the present study. A. *Amanita fuligineodisca*; B. *Amanita colombiana*; C. *Amanita sororcula*; D. *Amanita xylinivolva*; E. *Amanita flavoconia*; F. *Amanita brunneolocularis* var. *pallida*; G. *Amanita citrina*; H. *Amanita virosa*.

presence of a prominent basal bulb and appendiculate margin of the pileus in species within section *Lepidella*, differing from species in section *Phalloideae*, which lack these two characteristics in the stipe and the pileus, respectively (Jenkins 1986; Drehmel et al. 1999).

Following the taxonomic keys (Tulloss et al. 1992; Franco-Molano et al. 2000; Tulloss 2002, 2008, 2009a; Halling and Mueller 2005), 27 basidiomes were classified within subgenus *Lepidella*: 24 in section *Validae* and 3 specimens in section *Phalloideae*. Individuals classified within section *Validae* were recognized for having even and nonappendiculate pileus margins, globose to ellipsoid spores, and membranous to friable volva, which, according to Bas (1969), corresponds microscopically to the presence of inflated terminal cells in the universal veil. Thirteen basidiomes were determined to be *A. flavoconia* (FIG. 1E), characterized by their yellow orange pileus, pale yellowish nonbruising stipe, and a mean spore Q' = 1.21. Three samples represented the species *A. flavoconia* in the phylogeny, which grouped within a cluster of species that lacks red bruising reactions on the stipe (FIG. 2, clade j). In contrast, nine specimens were classified in the rubescent group (FIGS. 2 and 3A, clade k), *A. brunneolocularis* var. *pallida*, var. nov. (FIG. 3A, clade q), and *A. rubescens* (FIG. 3A, clade r). Two rubescent collections associated with *Pinus patula* plantations were determined as *A. rubescens* according to the tabular key of rubescent taxa (Tulloss 2002) and are characterized by having a brown pileus with pale margins, a cream-colored annulus, and spores L' = 8.75 µm with Q' = 1.43. On the other hand, two collections associated with *Q. humboldtii* were determined as *A. brunneolocularis* following Tulloss et al. (1992) and Tulloss (2002).

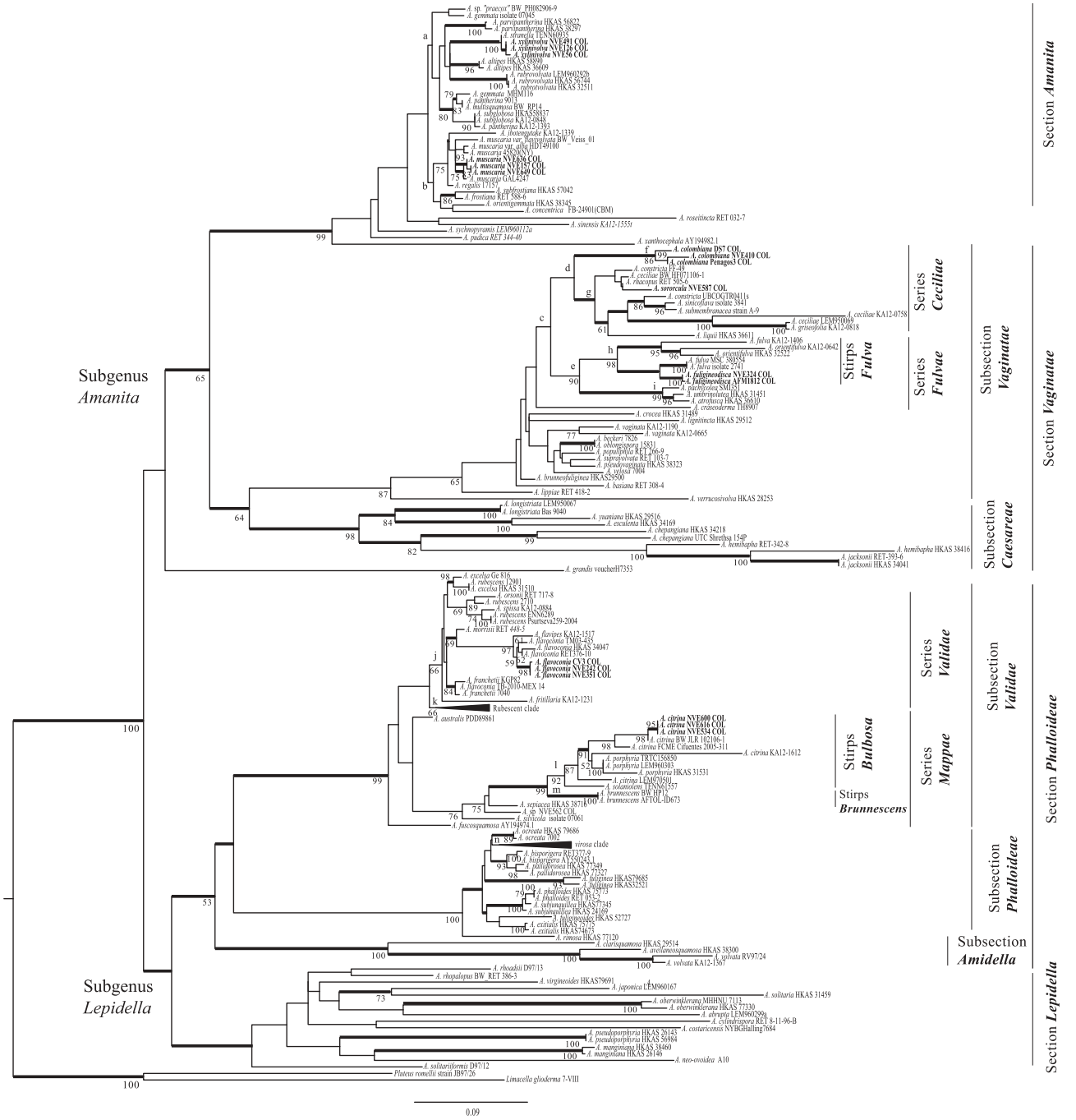

Figure 2. Phylogenetic reconstruction of three concatenated loci: 28S, ITS, and *TEF1* gene, using Bayesian and ML inferences. Bayesian posterior probability (PP) values are shown as thick black branches, indicating a PP >0.95. ML bootstrap values (BS) >75% are indicated above each branch.

Furthermore, following the taxonomic key of Tulloss (2005a), two basidiomes were classified as *A. citrina* in the section *Validae*, and three basidiomes classified as *A. virosa* (FIG. 3B) in the section *Phalloideae*.

In the following Taxonomy section, new records for Colombia are described. The term "undifferentiated hyphae," following Tulloss et al. (1992), means the presence of thin-walled hyphae lacking refractive contents. A detailed description of the species *A. colombiana, A. flavoconia, A. fuligineodisca, A. muscaria, A. rubescens, A. sororcula*, and *A. xylinivolva* is provided by Tulloss et al. (1992), Franco-Molano et al. (2000), and Halling and Mueller (2005).

TAXONOMY

Amanita brunneolocularis Tulloss, Ovrebo & Halling 1992 var. ***brunneolocularis***

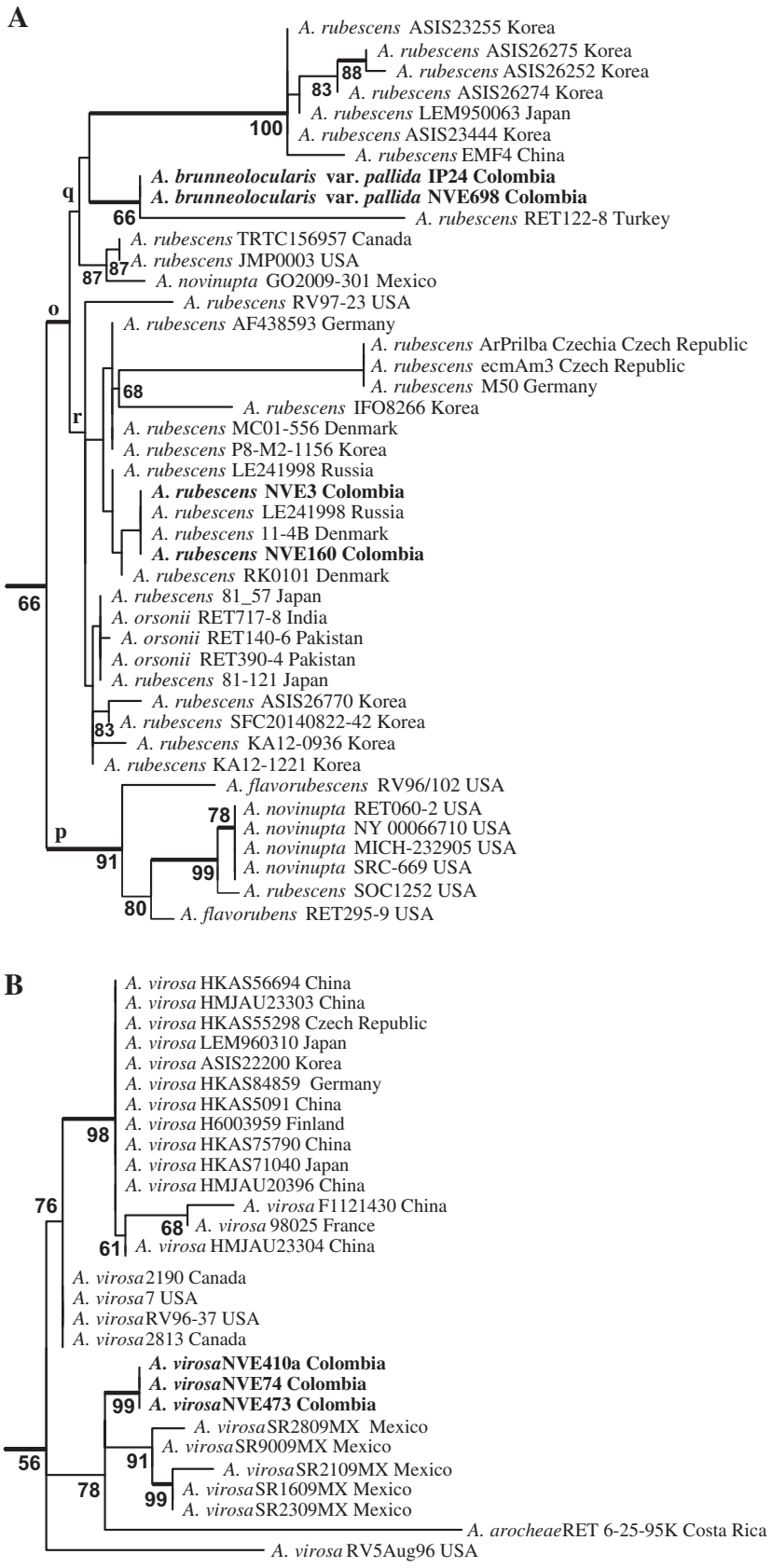

Figure 3. A. Rubescent clade from FIG. 2, clade k. B. *virosa* clade extracted from FIG. 2, clade n.

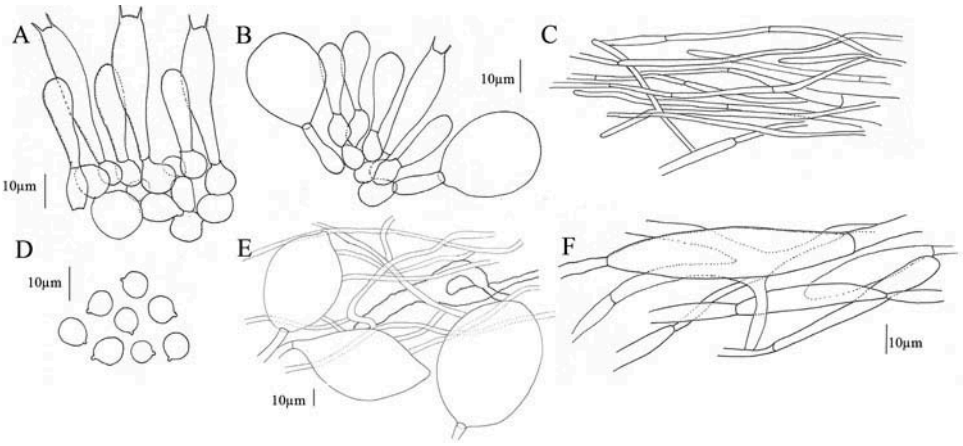

Figure 4. Micrographs of *Amanita brunneolocularis* var. *pallida* structures. A. Basidia; B. Cheilocystidia; C. Suprapellis; D. Spores; E. Volval elements; F. Subpelli.

This variety corresponds to the species described by Tulloss et al. (1992), which is the type variety for *A. brunneolocularis*.

Amanita brunneolocularis Tulloss, Ovrebo & Halling 1992 var. ***pallida***, var. nov. N. Vargas FIGS. 1F; 2, clade k; 3A, clade q; 4

MycoBank MB800647

Typification: COLOMBIA. BOYACA: Municipio de Villa de Leyva, Vereda Capilla, 5°39′26.78″N, 73°30′ 46.41″W, on soil in *Quercus humboldtii* forest, 24 Apr 2007, *Isabel Pulgarín 24 (IP24)* (**holotype** ANDES_F2268).

Etymology: Named after the pale color in the pileus, stipe, and inflated cells in the universal veil.

Diagnosis: The variety is macroscopically characterized by the grayish yellow to light orange pileus that is never white in young or mature specimens, and yellowish white to orange white, thin, irregular universal veil patches over the surface of the pileus. The stipe is pale orange and characterized by a subglobose bulb, a skirt-like, pale orange annulus, and a pinkish white friable volva with crumbling remnants about the stipe base. The stipe context contains oleiferous hyphae. The universal veil is formed by wide, colorless, inflated pyriform hyphae and contains oleiferous hyphae with pale yellow walls. The basidiospores are subglobose to broadly ellipsoid with a Q' = 1.1.

Pileus: 50–60 mm wide, conical to convex; surface even to finely fibrillose, grayish yellow (4B6) becoming light yellow (4A4) to light orange (5A4) to pale grayish yellow (4B4) toward margin; young or mature specimens never white; margin nonstriate, nonappendiculate; universal veil sometimes present over the surface of the pileus as irregular, thin, yellowish white (4A2) to orange white (5A2) or orange gray (6B2) patches. Context: 2–4 mm thick, white, unchanging. Lamellae: 4 mm wide, free, close, white; margin fimbriate; lamellulae showing different lengths. Stipe: 125–135 × 14–18 mm, central; surface fibrillose, white at apex, pale orange (6A3) to orange white (5A2); with subglobose bulb. Context not noted. Annulus: Superior, membranous, thin, skirt-like, pale orange (6A3), striate above, not totally detached from pileus margin. Volva: Pale orange (6A3) to pinkish white (7A2), sometimes with dull red (8B4) traces towards base, friable, leaving patches and crumbling remnants about the stalk base. Odor: Nondistinctive. KOH negative in pileus and stipe contexts.

Pileipellis: Light yellow-green in 3% KOH, as cutis of filamentous hyphae 7–15 µm wide, subradially arranged, some hyphae branching, gelatinized. Pileus context: Interwoven, undifferentiated hyphae often short, inflated, with yellow walls. Lamellar trama: Bilateral, with distinct width of the central stratum (wcs = 35–50 µm); formed by 8–10 µm wide, filamentous, undifferentiated, branching hyphae. Subhymenium: Composed of inflated ramose cells 10 µm × 10 µm between central stratum and base of basidia, broadly fusiform, with basidia arising from inflated cells, with a distance from an outer margin of the central stratum to the nearest base of a basidium (wst-near) = 10–13 µm and with a distance from an outer margin of the central stratum to the farthest base of a basidium (wst-far) = 18–30 µm. Lamellar edge cells: Globose inflated cells or sphaerocytes, 17–38 µm × 17–30 µm, with oleiferous hyphae at lower end of trama containing resinous material that turns yellow in 3% KOH. Basidia: (37–40)–45 × 8–(11–15) µm, four-sterigmate, with some basidioles present; clamps not observed. Stipe context: Longitudinally arranged; filamentous, undifferentiated hyphae 5–10 µm wide, with walls thin to slightly thickened, acrophysalids 50–110 × 20–26 µm; oleiferous hyphae 6–20 µm wide. Stipitipellis: Cutis with filamentous hyphae. Universal veil:

Formed by inflated piriform hyphae 80–140 µm long × 40–60 µm wide, presence of oleiferous hyphae 5–8 µm wide, with yellow walls. Partial veil: Formed by 45–60 × 10–20 µm, inflated, filamentous, branching hyphae, some with yellow content. Basidiospores: White in mass, [90/3/3] 7.0–10.0(11.5) × 7.0–9.0 µm (L = 8.1–8.8 µm; L' = 8.5 µm; W = 7.4–8.2 µm; W' = 7.8 µm; Q = 1.05–1.3; Q' = 1.1), hyaline, thin-walled, smooth, amyloid, subglobose to broadly ellipsoid; contents multiguttulate.

Material examined: COLOMBIA. BOYACA: Municipio de Arcabuco, Vereda Peñas Blancas, 5°48′54.6″N, 73°28′75.1″W, on soil in *Q. humboldtii* forest, 27 Apr 2014, NVE698; ANDES_F2201. BOYACA: Municipio de Arcabuco, 5°45′35.38″N, 73°26′47.10″W, on soil in *Q. humboldtii* forest, 15 Dec 2013, NVE624, ANDES_F2126.

Notes: This variety was determined to belong to the species *A. brunneolocularis* according to Tulloss et al. (1992) and Tulloss (2009a). It differs microscopically from the type species *A. brunneolocularis* var. *brunneolocularis* by the colorless inflated pyriform cells present in the universal veil; the spores in *A. brunneolocularis* var. *pallida* are subglobose with Q' = 1.1, whereas in *A. brunneolocularis* var. *brunneolocularis* are shorter and broadly ellipsoid with Q' = 1.3; the oleiferous hyphae in *A. brunneolocularis* var. *pallida* are common at the end of the lamella trama, the stipe context, and the universal veil, whereas in *A. brunneolocularis* var. *brunneolocularis* they are scarce or absent. The two varieties differ in the following macroscopic characteristics: the fibrils in the stipe of *A. brunneolocularis* var. *pallida* are pale cream bruising to pinkish cream, conversely in *A. brunneolocularis* var. *brunneolocularis* are white fibrils that become deep red to reddish brown to gray or black over a pallid ground; the pileus is pale cream yellow in *A. brunneolocularis* var. *pallida*, whereas in *A. brunneolocularis* var. *brunneolocularis* it is dull buff to reddish brown; *A. brunneolocularis* var. *pallida* has a pale orange volva, and *A. brunneolocularis* var. *brunneolocularis* has a dark brown volva.

Some close species to be compared with *A. brunneolocularis* var. *pallida* are *A. rubescens* var. *alba* Coker, *A. orsonii* Ash. Kumar & T.N. Lakh., and *A. novinupta* Tulloss and J. Lindgr: the spores in *A. rubescens* var. *alba* differ from those in *A. brunneolocularis* var. *pallida*, because they are more ellipsoid (Q' = 1.4) (Tulloss 2002), whereas in *A. brunneolocularis* var. *pallida* they are globose to subglobose; the pileus is white at first (Tulloss 2002) to cream with bronze stains in *A. rubescens* var. *alba* and grayish yellow to light orange (but not white) in young or mature specimens in *A. brunneolocularis* var. *pallida*; the universal veil in *A. rubescens* var. *alba* is pale yellow to cream, and pale cream to pink in *A. brunneolocularis* var. *pallida*. The species *A. orsonii* differs from *A. brunneolocularis* var. *pallida* by a pileus that is white at first (Tulloss 2002), staining orange (Tulloss 2009c), turning to shades of red or brown with handling or aging, whereas in *A. brunneolocularis* var. *pallida* the pileus is grayish yellow to light orange (not white) at first; the stipe is grayish red to grayish brown in *A. orsonii*, whereas in *A. brunneolocularis* var. *pallida* it has fibrils that are pale cream to pink; in *A. orsonii*, the universal veil has grayish white to grayish red to reddish brown patches (Tulloss 2009c), whereas in *A. brunneolocularis* var. *pallida* the universal veil is pale orange; *A. orsonii* lacks oleiferous hyphae in the universal veil (Tulloss et al. 2001); the spores are broadly ellipsoid to ellipsoid in *A. orsonii*, whereas in *A. brunneolocularis* var. *pallida* the spores are globose to subglobose. *Amanita novinupta* differs from *A. brunneolocularis* var. *pallida* and the above species in the presence of subpyramidal warts in the universal veil; *A novinupta* lacks oleiferous hyphae in the universal veil, whereas *A. brunneolocularis* var. *pallida* contains oleiferous hyphae; the pileus in *A. novinupta* is white at first then white with a pink tint; *A. novinupta* has ellipsoid spores (Tulloss and Lindgren 1994; Tulloss 2002), and *A. brunneolocularis* var. *pallida* has globose to subglobose spores. According to the classification of rubescent taxa (Tulloss 2002), the new variety differs from the taxa in the rubescent group by a smaller Q' = 1.1, which corresponds to more subglobose spores, than in other rubescent taxa with larger Q' values.

The BLAST search of the sequences corresponding to collections IP24 and NVE698 in the National Center for Biotechnology Information (NCBI) and the UNITE databases showed 98% identity with a maximum score of 869, with the sequence corresponding to *A. rubescens* voucher LE241998 (accession number JF313652). The concatenated phylogeny showed two different groups within the rubescent clade: the first clade (FIG. 3A, clade q) includes *A. rubescens* specimens from Japan, Korea, China, and Turkey, together with *A. brunneolocularis* var. *pallida* collected in *Q. humboldtii* stands. The concatenated and ITS trees recovered *A. brunneolocularis* var. *pallida* close to *A. rubescens* from Asia (FIG. 3A; SUPPLEMENTARY FIG. 2). A second clade (FIG. 3A, clade r) includes *A. rubescens* from *P. patula* plantations, morphologically assigned to *A. rubescens* close to European collections. A detailed morphological

revision of the specimens of the rubescent clade (FIG. 3A, clade o) would allow testing if they could be assigned to new taxa in light of these results.

Amanita citrina Pers. 1797 FIGS. 1G; 2, clade l

Pileus: 48–52 mm wide, convex to hemispheric; surface dry, bright, smooth, with some patches of pale brown membranous universal veil, pastel green (30A4) to pale green (30A3); margin even. Context: 6–7 mm thick, white, unchanging. Lamellae: 4.2–4.5 mm broad, free to slightly adnexed, close, pale green (30A3). Stipe: 70–80 mm long × 11 mm wide at the upper half and 40 mm in the bulbose base, fibrillose to slightly rugose in the upper half to fibrillose opaque at the lower half, compact with thin hollow line, upper half concolorous with the pileus, the lower half pale green with traces of light orange (5A4), abruptly bulbous, and marginate basal bulb; context cream. Annulus: Pale green (30A3), pendant, skirt-like ring. Volva: Limbate to membranous, pale orange (5A49) to grayish orange (5B4), becoming gelatinous and putrescent dark with age. Odor: Potato-like. KOH negative in pileus and stipe contexts. Pileipellis formed by filamentous, inflated, and periclinally arranged hyphae. Hymenophoral trama: Bilateral, with distinct central stratum (wcs = 93–107 µm); formed by 7–10 µm wide, filamentous, undifferentiated, branching hyphae; hyaline, inamyloid. Universal veil: Formed by 5–8 µm wide narrow hyphae, occasionally 85–100 µm long × 40–65 µm wide inflated hyphae; and oleiferous hyphae. Basidiospores pale cream in mass [60/2/1]: white in mass, 6–10(–11) µm long, 8–11 µm broad, mean Q' = 1.3, subglobose, smooth, slightly amyloid. Basidia: (30–)42–46 µm long, (5–)7–10 µm broad, four-sterigmate basidia.

Material examined: COLOMBIA. BOYACÁ: Municipio de Arcabuco, 5°45′35.38″N, 73°26′47.10″W, on soil in *Quercus humboldtii* forests, 15 Dec 2013, Natalia Vargas Estupiñan 600 (NVE600), ANDES_F2101; Natalia Vargas Estupiñan 616 (NVE616), ANDES_F2117.

Notes: Collections of *A. citrina* (FIG. 1F) were classified following the taxonomic keys corresponding to North American and Canadian species (Tulloss 2005a). The key provided for species from Costa Rica and neighboring regions (mostly associated to neotropical *Quercus* spp.) was not used, since it does not include this species (Tulloss 2009a). Collections NVE600 and NVE616 (FIG. 1G) were classified on the basis of the pale yellowish pileus, marginate basal bulb, a distinct potato odor, strongly limbate volva, and broadly ellipsoid spores (Q′ = 1.3) (Tulloss 2005a). Individuals of *A. citrina* from Colombia were grouped phylogenetically closer to worldwide collections of *A. citrina*, in the group *Mappae*, where species are distinguished by having limbate volva, with marginate to submarginate basal bulb (Singer 1986; Drehmel et al. 1999) (FIG. 2, clade l). These collections of *A. citrina* associated with *Q. humboldtii* constitute the first report for Colombia of a species allocated in stirps *Bulbosa*.

Amanita virosa Bertill. 1866 FIGS. 1H; 2, clade n; 3B; 5

Pileus: 50–80 mm wide, convex to hemispheric; surface dry, smooth, white (1A1) with yellowish white (1A2), membranous universal veil patches, white (1A1) towards the margin; margin smooth to slightly appendiculate. Context: 4 mm thick, white, unchanging. Lamellae: 3.5 mm, broad, free to adnexed, close, yellow white (3A2). Stipe: 90 mm long × 6 mm wide at the upper half and 28 mm at the lower half, fibrillose, hollow, white towards the apex to yellow white (1A2) towards the base, abruptly bulbous; context white. Annulus: Subapical, skirt-like, thin. Volva: Limbate to membranous, white. 3% KOH is slightly pale yellow in the pileus surface. Pileipellis suprapellis formed by filamentous, periclinally arranged, very thin hyphae, 2–3 µm wide, with thin wall, oleiferous hyphae of 5–6 µm wide; subpellis with inflated hyphae. Hymenophoral trama: Divergent, hyaline, inamyloid. Universal veil: Formed by thin-walled, narrow, occasionally slightly inflated hyphae. Basidiospores: White in mass, [90/3/3], 7–10(–11) µm long, 8–10(–11) µm broad, mean Q' = 1.03, globose, smooth, slightly amyloid. Basidia: 36–44 (–47) µm long, (11–)14–16 µm broad, four-sterigmate basidia.

Material examined: COLOMBIA. BOYACÁ: Municipio de Arcabuco, Vereda Peñas Blancas, 5°48.546″N, 73° 28.751″W; on soil in *Q. humboldtii* forest, Natalia Vargas Estupiñan 410a (NVE410a), ANDES_F2272. BOYACÁ: Municipio de Villa de Leyva, Vereda Capilla, 5°39′26.78″ N, 73°30′46.41″W, on soil in *Q. humboldtii* forest, NVE74 (ANDES_F239). BOYACÁ: Municipio de Saboyá, Garavito, 5°42.006″N, 73°46.170″W; on soil in *Q. humboldtii* forest, Natalia Vargas Estupiñan 473 (NVE473), ANDES_F973.

Notes: The collections NVE74, NVE475, and NVE410a are morphologically similar to *A. arocheae* described by Tulloss et al. (1992) but differ in having a white pileus and stipe. The ITS sequence FJ890028.1 representing the collection NVE74 was previously included in the study of lethal *Amanita* spp. by Cai et al. (2014), interpreting the collection (accession number FJ890028) as *A. bisporigera*. However, characters such as the four-sterigmate basidia (FIG. 5A) and globose spores (Q = 1.03–1.1) suggest that this collection does not represent *A. bisporigera* but *A. virosa*. In a previous study (Vargas et al. 2011), the collection NVE74 was analyzed by its toxin composition, associated with the group of lethal *Amanita* by a high concentration of phallotoxins. *A. virosa* is mainly reported

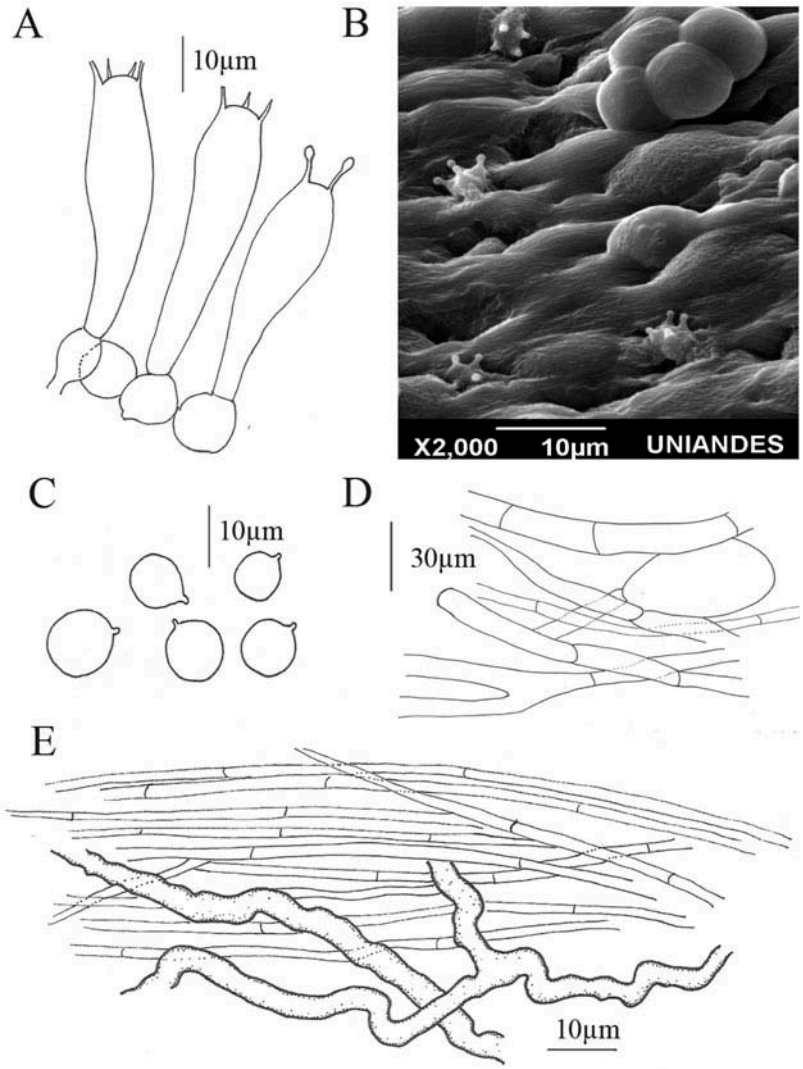

Figure 5. Micrographs of *Amanita virosa* structures. A. Basidia; C. Spores; D. Volval elements; E. Suprapellis. B. Electronic microscopy of the hymenial layer showing four-sterigmate basidia.

in Palearctic regions—Europe and northeastern Asia (Zhang et al. 2010; Cai et al. 2014). This would be the first report of this species growing under *Quercus humboldtii* in Colombia. The collections representing this taxon clearly grouped closer to *A. virosa* collections from Mexico (SUPPLEMENTARY FIGS. 2 and 3) within subsection *Phalloideae* sensu Drehmel et al. (1999) (FIG. 2, clade n).

DISCUSSION

Previous phylogenetic studies assessed by ML and BI methods have supported the monophyly of two subgenera *Amanita* and *Lepidella* (Drehmel et al. 1999; Justo et al. 2010; Wolfe et al. 2012; Cai et al. 2014; Hosen et al. 2015), whereas other phylogenetic hypotheses assessed by distance-based methods or parsimony have failed to support the monophyly of the two subgenera (Weiss et al. 1998; Oda et al. 1999; Zhang et al. 2004). Our results were not conclusive on the monophyly of each subgenus, with only *Lepidella* having high support from the BI analysis.

The infrasubgeneric classification varies among studies regarding morphological characters and molecular markers (Weiss et al. 1998; Oda et al. 1999; Zhang et al. 2004). A previous phylogenetic study by Oda et al. (1999) with ITS and 28S showed no clear grouping of species in section *Amanita* within subgenus *Amanita*. More recently, Zhang et al. (2004) recovered section *Caesareae* and section *Vaginatae* as sister groups, with higher support values with the ITS region but not with the 28S region. So far, our phylogenetic hypotheses with ML and BI corroborate that, within subgenus *Amanita*, two well-supported sections are delimited: *Amanita* and *Vaginatae* (FIG. 2). Furthermore, the section *Vaginatae* was divided into two subsections, which agrees with the infrageneric classification system

sensu Drehmel et al. (1999). The main morphological character that could support splitting these two subsections is the presence of an inconspicuous basal bulb and normally the absence of a ring in subsection *Vaginatae*. Besides that, the rank of subsection for the *Caesareae* group is consistently shown in our phylogenetic hypothesis (FIG. 2).

Our results show high BI support for the monophyly of sections *Phalloideae* and *Lepidella* sensu Drehmel et al. (1999) (FIG. 2) within the subgenus *Lepidella*. Subsections *Phalloideae* and *Validae* form the sister group of subsection *Amidella*, agreeing with Weiss et al. (1998). The monophyly of subsection *Amidella* in our phylogenetic tree was well supported and grouped species characterized by a thick and firm saccate volva and elongated to cylindrical spores (Singer 1986), as opposed to species with saccate to membranous volva and not elongated spores, grouped in subsection *Phalloideae*, and to those with a poorly developed volva in subsection *Validae*. In addition, the division of species in subsection *Amidella* could be further supported by the presence of the appendiculate character of the pileus margin, considered a more basal character than even or nonstriate margins of the pileus (Bas 1969).

Determining taxon ranks associated to particular names, i.e., species names, will normally differ between traditional classification systems and phylogenetic hypotheses (Cantino and de Queiros 2010). The phylogenetic framework proposed by Drehmel et al. (1999) provides a classification system that agrees with the three-gene phylogenetic approach in the present study. However, taxonomic keys used in this study propose ranks that differ from some of the ranks displayed in the phylogeny. We compared different classification sources at the infrageneric rank (TABLE 1).

Our phylogenetic approach allows us to go below the subsection lever in classification, since not many taxonomic keys treat the ranks below this level. Some series as well as stirps in the monographic treatment for the family Amanitaceae in www.amanitaceae.org can be assigned to clades in our proposed phylogeny. Two clades within subsection *Vaginatae* correspond to the series *Ceciliae* (FIG. 2, clade d) and *Fulvae* (FIG. 2, clade e) (Tulloss and Yang 2012a, 2012b). The *A. fuligineodisca* group share features with the species *A. fulva* such as the Q' value of 1.03 obtained in *A. fuligineodisca* specimens, closer to that in *A. fulva* (Jenkins 1986), but they differ in a darker color of the pileus in the disc to pale brown margins and a lower angle of split of the inflated cells of the hymenophoral trama in *A. fuligineodisca* (Tulloss et al. 1992). This species also differed from the species *A. humboldtii* because of the dark brown to blackish brown center to pale brown margin of the pileus.

Moreover, a more specific division separates the specimens classified as *A. colombiana* (FIG. 2, clade f). Following the taxonomic key provided in Tulloss et al. (1992), three specimens were characterized by a pulverulent reddish volva, which were determined as *A. colombiana*, and differed from specimens characterized by a membranous volva in shades of gray (FIG. 2, clade g), including the species *A. sorocula* characterized by the submembranous grayish volva, thick smoky gray membranous remnants over the pileus surface, and densely sulcate pileus margin (FIG. 1C).

The concatenated tree shows two groupings (FIG. 2, clades j and k) within subsection *Validae*, series *Validae*. Clade j consisted of species characterized by a nonrubescent color in the stipe and shades of yellow at the pileus. In contrast, clade k contained the rubescent taxa *A. brunneolocularis, A. novinupta, A. orsonii*, and *A. rubescens*, characterized by fuscous to brown pileus in shades of red over the disc and pale brown towards the margin. The new variety *A. brunneolocularis* var. *pallida*, characterized by the colorless pyriform cells in the volva, the subglobose to broadly ellipsoid spores, and the pale colors in the pileus and stipe, was positioned in series *Validae* (FIG. 2, clade k; FIG. 3A). Our results show that the species *A. rubescens* is paraphyletic (FIG. 3A). A specimen classified as *A. rubescens* from Turkey forms a highly supported clade with the new variety *A. brunneolocularis* var. *pallida* (FIG. 3A, clade q). *A. novinupta, A. orsonii*, and *A. flavorubescens* are also nested with *A. rubescens* collections (FIG. 3A, clades p, r). This fact raises the question of whether more than one species has been incorrectly assigned to *A. rubescens*, and a thorough morphological and phylogenetic revision of this group should be made to acknowledge their phylogenetic and morphological diversity. On the other hand, the subsection *Validae*, series *Mappae*, showed two distinct well-supported clades corresponding to the stirps *Brunnescens* (Tulloss and Yang 2015b; FIG. 2, clade l) and the stirps *Bulbosa* (Tulloss and Yang 2015b; FIG. 2, clade m).

Finally, the similarity parameter, proposed by Tulloss (2005b), among species present in Colombia and those in other regions such as Costa Rica or North America, will tend to change as studies of local macrofungal diversity increase. For instance, the similarity parameter is likely to increase after adding *A. rubescens* and *A. citrina* to the Colombian species list in subsection *Validae*, when compared with countries such as Costa Rica and the United States. In addition to the 16 previously reported species, two new reports, *A.*

citrina and *A. virosa*, are included in this study for a total of 18 species currently reported in Colombia. Although the phylogenenetic position of 10 species was assessed in the present study, from specimens collected between 2012 and 2014 in the northeastern Andean forests of Colombia, future phylogenetic analysis should include more species by analyzing early herbaria collections and/or sampling more areas across the Andean montane forests in Colombia. Moreover, in order to have a broader panorama of the diversity of the genus in Colombia, it will be necessary to include species that are distributed in lowland tropical forests, where a high diversity of *Amanita* spp. is expected (Simmons et al. 2001; Henkel et al. 2012).

In the present study, the phylogenetic relationships of *Amanita* species found in Colombia was assessed by combining classical morphological and molecular methods with character-based methods such as maximum likelihood and Bayesian inference and comparing with a wide number of species distributed worldwide. This study led to the description of a new variety and a new record, together with an analysis of the phylogenetic relationship of species that were not previously included in the *Amanita* phylogeny. Furthermore, it allowed classification at a lower rank (e.g., stirps), permitting a better understanding of system classifications of the collected species. This combined morphological and phylogenetic approach can be useful to avoid ambiguous classifications of the taxa when these classifications are based just on one criterion.

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