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Helvella jocatoi sp. nov. (Pezizales, Ascomycota), a new species from *H. lacunosa* complex with cultural importance in central Mexico *Abies religiosa* forests

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Abstract

Helvella lacunosa is a species complex, with *Helvella lacunosa* s.s. not currently distributed in America. The objective of this study was to resolve the taxonomy of specimens from central Mexico identified as *Helvella lacunosa* s.l. associated with *Abies religiosa* forests. The nuclear ITS and LSU regions were PCR-amplified and sequenced from dry herbaria specimens. Phylogenetic analyses were based on Parsimony, Maximum Likelihood and Bayesian Inference approaches. Sequences of *Helvella* from *A. religiosa* forests grouped into a well-supported lineage within the North American clade together with *Helvella dryophila* (associated with *Quercus* in western USA) and *Helvella vespertina* (associated with conifer forests in western USA). Therefore, we describe and illustrate *Helvella jocatoi* as a new species based on phylogeny and morphological traits. In central Mexico, this taxon is an edible mushroom known as "gachupín", has high cultural importance and is sold in large quantities. The description of this new species restricted to *A. religiosa* forests has implications for its conservation since its habitat is endangered.

Keywords: *Abies religiosa*, Ecology, *Helvella lacunosa* complex, Phylogeny, edible mushrooms

Introduction

The genus *Helvella* L. is a monophyletic lineage with 83 species whose phylogeny (Landeros *et al.* 2015, Skrede *et al.* 2017) and diversity (Landvik *et al.* 1999, Nguyen *et al.* 2013, Ariyawansa *et al.* 2015, Zhao *et al.* 2015, Wang *et al.* 2016, Zhao *et al.* 2016) are under continuous research. Taxonomic studies performed before DNA sequencing assumed that European species as *H. crispa* (Scop.) Fr. or *H. lacunosa* Afzel. are also distributed in America and Asia (Dissing 1966, Weber 1972, Abbott & Currah 1997, Zhuang 2004). However molecular phylogenies have demonstrated that their distribution is continentally restricted and they constitute species complexes (Nguyen *et al.* 2013, Ariyawansa *et al.* 2015, Landeros *et al.* 2015, Zhao *et al.* 2015, Wang *et al.* 2016, Zhao *et al.* 2016, Skrede *et al.* 2017). Additionally, *Helvella* has been intensely studied because of the edibility of many species (Lara-Vázquez *et al.* 2013, Thomé-Ortíz 2016, Zhao *et al.* 2016).

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Mushrooms determined as *H. lacunosa* in Mexico have wide reproductive seasonality fruiting from July to October (Burrola-Aguilar *et al.* 2013). These mushrooms are gathered by several indigenous communities like "otomíes" (Burrola-Aguilar *et al.* 2012, Lara-Vázquez *et al.* 2013), "matlatzincas" (Thomé-Ortíz 2016), "mazahuas" (Farfán *et al.* 2007) and "mixtecos" (Hernández-Santiago *et al.* 2016). People in rural communities name *H. lacunosa s.l.* mushrooms in Spanish as "gachupín negro" (Montoya *et al.* 2008), "gachupines", "hongo de carbón", "hongo negro", "soldaditos" (Burrola-Aguilar *et al.* 2012, Lara-Vázquez *et al.* 2013, Franco-Maass *et al.* 2016), "gachupi", "cuatlil", "charrito negro" (Pacheco-Cobos *et al.* 2015), "chicharrón" (Pérez-Moreno *et al.* 2008), "oreja", "orejita", "oreja negra", and "oreja de Judas" (Contreras-Cortés *et al.* 2018). They also receive names in original languages as "cjeshirgo" in "mazahua" (Farfán *et al.* 2007) and "xi'i kue ìe" in "mixteco" (Hernández-Santiago *et al.* 2016).

Helvella lacunosa s.l. has high cultural importance in the mountains of central Mexico (Burrola *et al.* 2012, Franco-Maass *et al.* 2016, Thomé-Ortíz 2016). To collect these mushrooms for commercial purposes, gatherers walk 3 to 8 hours into steep hills and tolerate harsh climate (Franco-Maass *et al.* 2016). Despite this, *H. lacunosa s.l.* is among the most sold mushrooms in traditional markets from central Mexico (Burrola *et al.* 2012, Lara-Vázquez *et al.* 2013, Pérez Moreno *et al*, 2008) at prices around $5.00 USD for half kilogram (Arteaga-Martínez & Moreno-Zárate 2006, Burrola-Aguilar *et al.* 2012). *Helvella lacunosa s.l.* mushrooms are dried to be consumed at the "Day of the dead" holiday—the dry sporomes are "offered" as food to the spirits of dead people—, or in Christmas holidays (Burrola-Aguilar *et al.* 2012, Lara-Vázquez *et al.* 2013, Thomé-Ortíz 2016). Also, *H. lacunosa s.l.* has pharmacological potential (Hernández-Santiago *et al.* 2016) since its δ-tocopherol and lycopene contents (Leal *et al.* 2013).

Nguyen *et al.* (2013) determined that *H. lacunosa s.s* is not distributed in North America and is a species complex composed by, at least five species. They described *H. dryophila* N.H. Nguyen & Vellinga and *H. vespertina* N.H. Nguyen & Vellinga from western USA and recognized at least three unnamed lineages from Mexico. Within this species complex, Ariyawansa *et al.* (2015) described *H. pseudolacunosa* Q. Zhao & K.D. Hyde and *H. rugosa* Q. Zhao & K.D. Hyde from China. Mushrooms species described in the *H. lacunosa* species complex are associated with *Pinus* and *Quercus* forests; in contrast, we focused on *H. lacunosa*-like specimens collected from *Abies religiosa* (Kunth) Schltdl. & Cham forests in central Mexico. Our objective was to resolve their phylogenetic relationships and to describe this species.

Material and methods

Samples and morphology

Dried specimens labeled as *Helvella lacunosa* and collected from *Abies religiosa* forests were obtained with permission from the herbaria: XAL, "Instituto de Ecología"; IBUG, "Instituto de Botánica de la Universidad de Guadalajara"; MEXU, "Instituto de Biología de la Universidad Nacional Autónoma de México".

Dried material was rehydrated and analyzed under compound microscope as described by Landeros *et al.* (2012). Briefly, hand sections were mounted in water or Melzer's reagent and measurements of spores, asci, paraphysis, width of subiculum, were registered. Also, the macromorphological data with taxonomic relevance were recorded. Color terms used the terminology of Kornerup & Wanscher (1967).

Molecular procedures

DNA was extracted and PCR-amplified with DNA Extract-N-Amp Plant PCR kit (Sigma-Aldrich, St. Louis, MO). PCR conditions were the same of Nguyen *et al.* (2013) and Landeros *et al.* (2015) with the following primers: ITS1 and ITS4 (White *et al.* 1990) for the ribosomal internal transcribed spacers (ITS) and LSU1 and LSUR2 or LROR and LR5 (Vigalys & Hester 1990) for the nuclear large ribosomal subunit (LSU). PCR products were sequenced in both directions with PCR primers with Sanger chemistry in "Laboratorio de Microbiología Molecular" (LAMIMO), "Laboratorio Nacional de Genómica para la Biodiversidad" (LANGEBIO), and "Laboratorio de Biología Molecular de la Biodiversidad y la salud" (LANABIO). Sequences were edited in Chromas Pro Ver. 1.41 (Technelysium, Australia) and submitted to Genbank (Table 1).

TABLE 1. DNA sequences used for phylogenetic analyses of *Helvella lacunosa* complex.

Species/vouchers		Genbank accession		Reference
		ITS	LSU	
		KC122839	KC122795	Nguyen *et al.* 2013
		KC122840	KC122794	Nguyen *et al.* 2013
Helvella dryophila		KC122828	KC122793	Nguyen *et al.* 2013
		KC122811	KC122772	Nguyen *et al.* 2013
		KC122831	KC122792	Nguyen *et al.* 2013
H. fusca			KY773101	Skrede *et al.* 2017
	AR09690	KC016121		Nguyen *et al.* 2013
	Castro-Castro7	MT334418	JX993068	Here ITS
	CB08326	KC016115*	MH399851*	Here LSU
	CB08331	KC016122		Nguyen *et al.* 2013
	CB08367	KC016114	MH399852	Here LSU
	GO-2009-088	KC016119		Nguyen *et al.* 2013
H. jocatoi	HC-PNNT-149	KC016117		Nguyen *et al.* 2013
	HC-PNNT-266	KC016116	MH399855	Here LSU
	Landeros3581	MT334416	MT334419	Here ITS and LSU
		KC122808	KC122770	Nguyen *et al.* 2013
		KC122809	KC122771	Nguyen *et al.* 2013
		KT894823	KT894830	Wang *et al.* 2016
H. lacunosa		KT894824	KT894831	Wang *et al.* 2016
			KY773002	Skrede *et al.* 2017
			KY773152***	Skrede *et al.* 2017
			KY772988**	Skrede *et al.* 2017
H. pallescens			KY773137	Skrede *et al.* 2017
			KY772933	Skrede *et al.* 2017
H. palustris			KY773123	Skrede *et al.* 2017
			KY772914	Skrede *et al.* 2017
H. philonotis			KY773086	Skrede *et al.* 2017
H. pseudolacunosa		KR493477	KT932630	Wang *et al.* 2016
		KR493476*	KT932629*	Wang *et al.* 2016
H. queletiana			KY773151**	Skrede *et al.* 2017
H. rugosa		KR493478	KT932631	Ariyawansa *et al.* 2015
		KR493475*	KR493511*	Ariyawansa *et al.* 2015
		KT894826	KT894833	Wang *et al.* 2016
H. sublactea		KT894827	KT894834	Wang *et al.* 2016
		KT894825*	KT894832*	Wang *et al.* 2016
H. sulcata			KY773001***	Skrede *et al.* 2017
		KC122848	KC122785	Nguyen *et al.* 2013
		KC122846	KC122777	Nguyen *et al.* 2013
H. vespertina		KC122854	KC122778	Nguyen *et al.* 2013
		KC122845	KC122787	Nguyen *et al.* 2013
		KC122779	KC122849	Nguyen *et al.* 2013
H. zhongtiaoensis		JX462578	KR493484	Zhao *et al.* 2015
Helvella sp.	GO-2009-279	KC016118	MH399853	Here LSU
	HC-PNNT-059	KC016124	MH399854	Here LSU
	LGD9002	MT334417	JX993067	Here ITS

* Holotype, ** Neotype, *** Epitype.

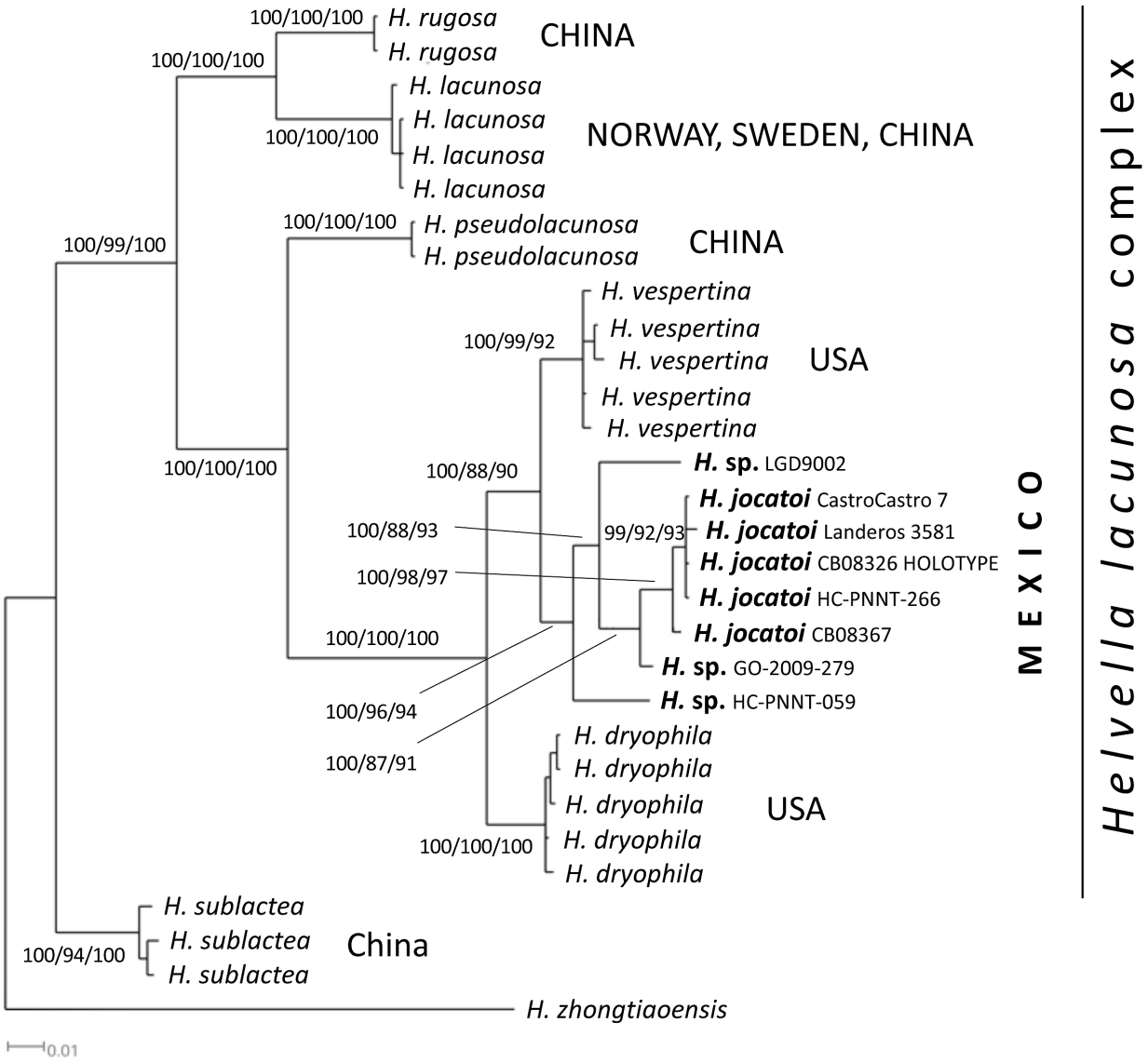

FIGURE 1. *Helvella lacunosa* complex phylogenetic analysis using an ITS and LSU concatenated alignment. Supports are posterior probability/bootstrap maximum parsimony/bootstrap maximum likelihood. Scale bar represents nucleotide changes per base.

Phylogenetic analyses

DNA sequences from Nguyen *et al.* (2013), Wang *et al.* (2013), Ariyawansa *et al.* (2015) and Skrede *et al.* (2017) were downloaded from GenBank (Table 1). ITS and LSU DNA sequences were aligned separately in MacClade 4.0 (Maddison & Maddison 2000) using *H. zhongtiaoensis* J. Z. Cao & B. Liu as outgroup and afterwards were concatenated. Ambiguous regions were deleted from the final DNA alignment.

The concatenated alignment was analyzed using Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI). MP analysis was done with PAUP 4.0a155 (Swofford 2002) using a heuristic search of 1000 replicates with the TBR algorithm for the branches exchange and 1000 bootstrap replicates for branch support (BMP). ML analysis was done with RAxML 7.2.6 (Stamatakis 2006) using the GTR+G substitution model and 1000 bootstrap replicates (BML). BI analysis was done in MrBayes 3.2.5 (Ronquist & Huelsenbeck 2003) with the GTR+G+I model suggested by jModeltest 3.7 (Posada & Crandall, 1998) under the following parameters: ngen=2000000, samplefreq=100 and nruns=4; and a burn-in of 25% to calculate the posterior probability (PP). The generation number corresponded with a standard deviation inferior to 0.01. As tree topology was the same in the three methods, we used the BI tree to represent all support values.

As some species in the *H. lacunosa* complex have only sequences for one locus (Table 1), each region was also analyzed separately with Bayesian Inference (BI) in MrBayes 3.2.5 (Ronquist & Huelsenbeck 2003). The substitution

model was GTR+G+I as suggested by jModeltest 3.7 (Posada & Crandall 1998) with the following conditions: ngen=1000000, samplefreq=100 y nruns=4.

Results

Phylogenetic analyses

In the concatenated phylogenetic analyses, Mexican samples from *A. religiosa* forests grouped in a strongly supported clade together with other *H. lacunosa* complex North American species (PP: 100; BMP: 100; BML: 100) (Figure 1). The sister species of all Mexican samples was *H. vespertina* (PP: 100; BMP: 88; BML: 90). When analyzed separately, ITS and LSU (Figure 2A, 2B) regions also found a strong support for the North American clade (ITS BMP: 100; LSU BMP: 100). Both analyses also found strong support for Mexican samples from *A. religiosa* forests (ITS BMP: 99; LSU BMP: 96). In all the analyses, three Mexican specimens (HC-PNNT-059, LGD9002, GO-2009-279) did not group with *H. jocatoi*, suggesting additional unnamed species in the *H. lacunosa* species complex from Mexico.

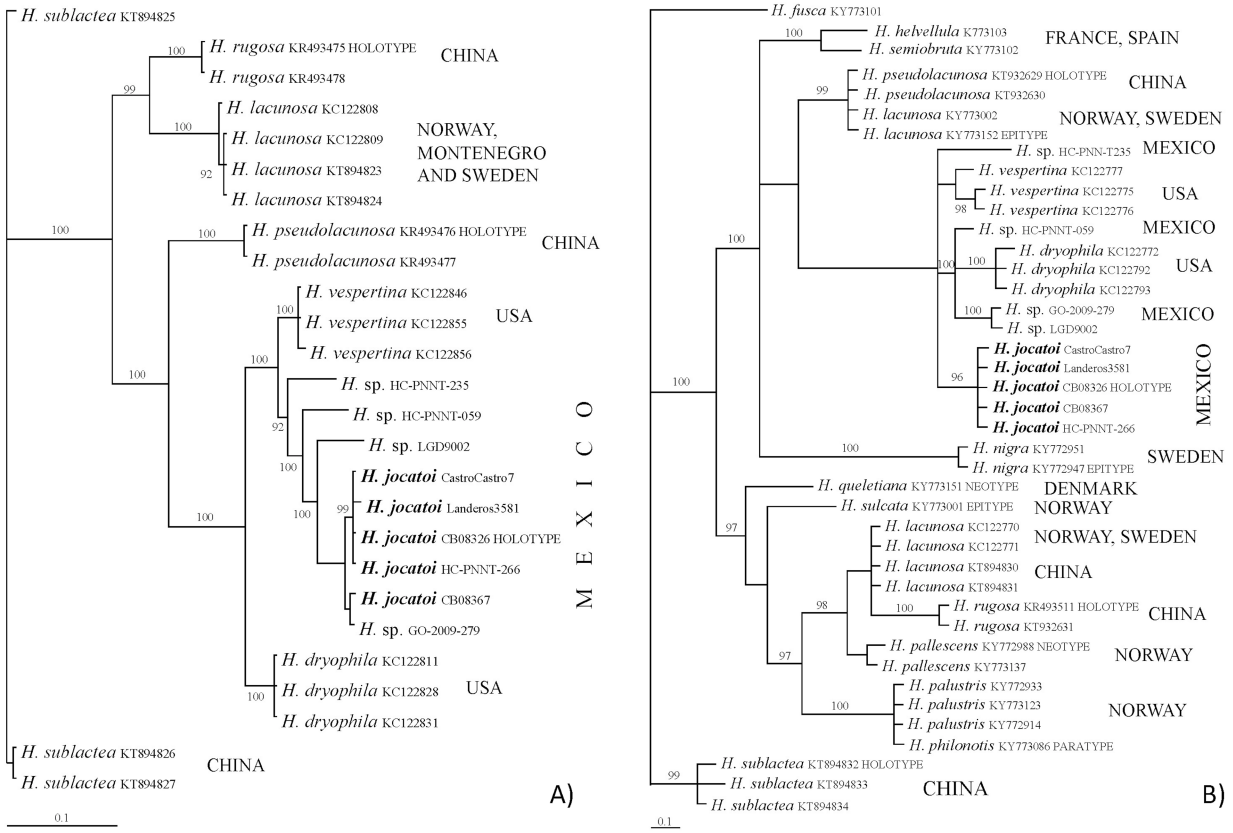

FIGURE 2. *Helvella lacunosa* complex phylogenetic analysis using ITS and LSU regions separately. A) ITS Bayesian phylogenetic analysis. B) LSU Bayesian phylogenetic analysis. Supports are posterior probability. Scale bar represents nucleotide changes per base.

Taxonomy

Helvella jocatoi F. Landeros, R. Garibay-Orijel & L. Guz.-Dáv. *sp. nov.* Figure 3
MycoBank 839202

Etymology: *jocato* (Spanish), is an acronym for the first two letters of the given name and two last names of José Castillo Tovar deceased at 2012 who was a prominent Mexican mycologist and teacher of the first author; *i* (Latin), pertaining to, indicates the genitive case in masculine form.

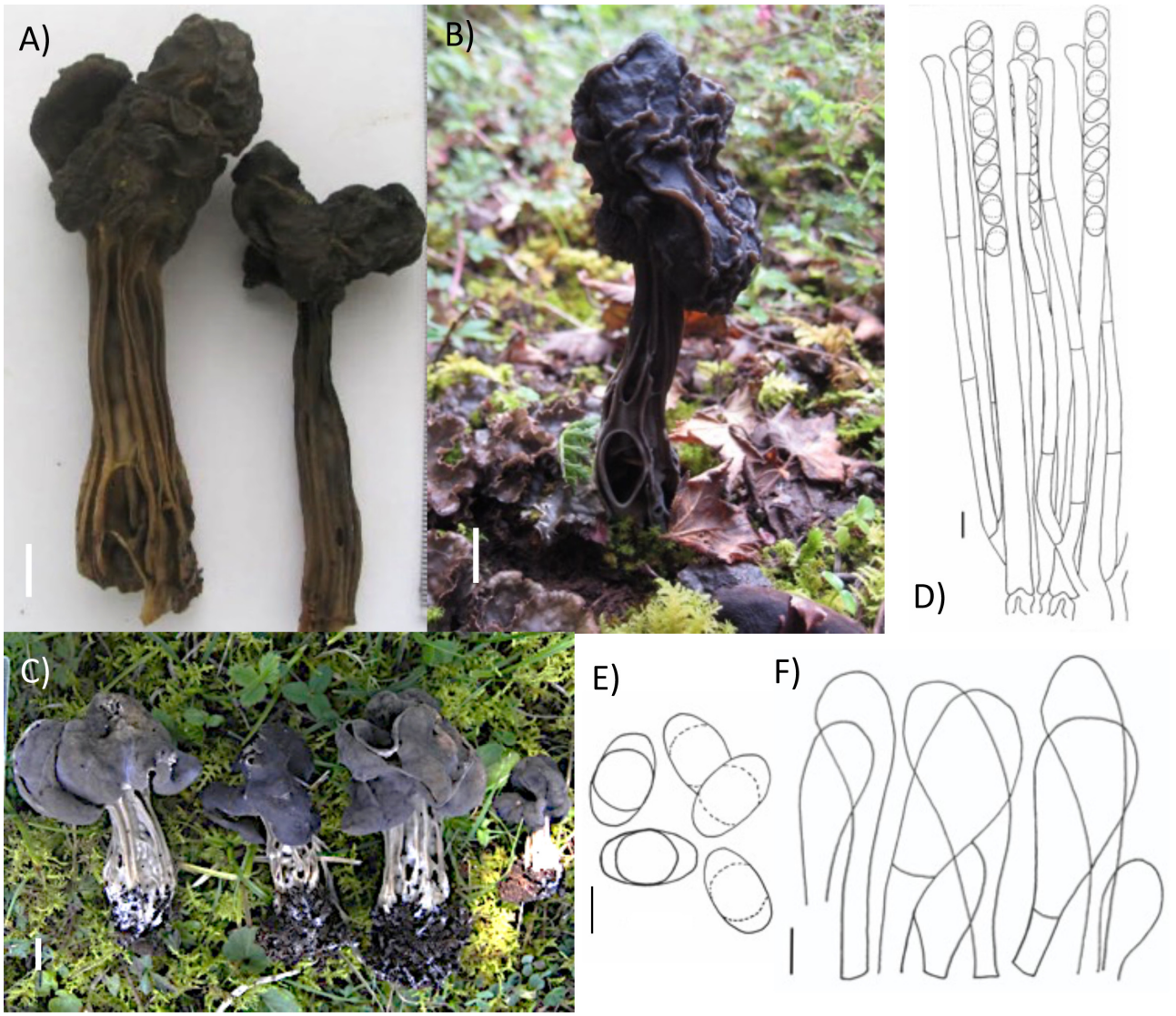

FIGURE 3. *Helvella jocatoi*. A) Holotype CB08326 (MEXU 25760); B) Castro-Castro 7 (IBUG); C) Landeros 3581 (IBUG); D) Hymenium; E) Ascospores; F) Ectal excipulum cells. A), B), C) Bars: 1 cm. D), E), F) Bars: 10 µm.

Holotype:—Mexico. Estado de México: Amanalco, Corral de piedra, September 21, 2009, CB08326 (MEXU 25760).

Diagnoses: Ascoma 40–160 mm high, pileus 20–60 mm, high 20–60 mm broad, with three lobes or irregularly lobed, dark gray to black. Pileus sterile surface smooth to slightly venous, white or slightly grayish. Stipe up to 120 mm high and up to 25 mm broad, cylindrical, lacunose, first white, then grayish even blackish. Ascospores 16.8–20.8 (–22) × 10.8–13 (–14) µm, hyaline. Solitary or in clusters, terrestrial or on mosses in *Abies religiosa* forests on the Trans Mexican Volcanic Belt.

Description: Ascoma 40–160 mm high. Pileus 20–60 mm high, 20–60 mm diam, with three lobes or irregularly lobed, dark gray to black, pileus board attached to stipe. Pileus sterile surface smooth to slightly venous, white to slightly gray. Stipe up to 120 mm high, 25 mm broad, cylindric, lacunose, white to gray at first, then almost black. Ascospores 16.8–20.8 (–22) × 10.8–13 (–14) µm, Q = 1.5–1.6, ellipsoidal, smooth when mature, hyaline, with one guttule. Asci 255–330 (–365) × 14–19 (–20) µm, not amyloid, pleurorhynchous with eight ascospores. Paraphyses cylindrical 3–6 µm wide to the half, apex 5–9 µm wide, with brown intracellular pigment. Ectal excipulum 60–80 µm, with cylindrical to narrowly clavate to clavate cells, 12–35 µm of length and 3.5–6 µm at apex. Slightly dextrinoid reaction in medullar excipulum and middle stipe tissue, others tissues are negative to Melzer's reactive.

Habit, habitat and phenology: Epigeous, solitary or aggregated in *Abies religiosa* forest on the Trans Mexican Volcanic Belt, on the ground or on moss during August to December.

TABLE 2. Comparison between *H. jocatoi* and closest species.

Characteristic	*H. lacunosa* s.s.	*H. jocatoi*	*H. pseudolacunosa*	*H. rugosa*	*H. dryophila*	*H. vespertina*
Apothecia or pileus size	30–60 × 20–45 mm	20–60 × 20–60 mm	10–40 × 20–30 mm	10–20 × 10–20 mm	15–45 × 20–45 mm	25–55(–150) × 25–50(–120) mm
Apothecia or pileus color	Black	Black greyish to all black	Grey to dark gray	Pale to dark gray or greyish brown	Dark gray-black, rarely pale gray	Light gray to dark gray
Stipe size	40–150 × 15–50(–60) mm	Up to 120 × 35 mm	20–80 × 7–15 mm	20–40 × 4–7 mm	35–75 × 10–20 mm	Up to 250 × 105 mm
Stipe color	Whitish to greyish	Whitish at first, black with age	Grey-white when young, becoming grey with age	Greyish Brown when young, becoming black when dry	Off-white when young, gray with age	White tuning to gray with age
Ascospores	17–19 × 9–11.5 µm	16.8–20.8(–22) × 10.8–13(–14) µm	(14–)15–19.5(–20) × (9.5–)10–12(–12.5) µm	(15–)15.5–18(–18.5) × (9.5–)10–11(–11.5) µm	15.7–19.6 × 9.8–12.7 µm	15.7–21.4 × 9.8–12.2(–13.7) µm
Vegetation	*Dryas*, subalpine birch forest, *Larix gmelinii*	*Abies religiosa* forests	*Larix gmelinii* and *Pinus koraiensis*	Deciduous *Quercus* sp. forests	Mixed *Quercus* forests	*Pinus*, *Pseudotsuga* and possibly *Abies* and *Arbutus*
Distribution	Boreal Europe	Central Mexico	China	China	Western North America	Western North America
Season	July to October	August to December	Around August	Around August	Mid-December to end of May	October-March (rarely in April)

Specimens examined: Mexico, Estado de Mexico: Amanalco, San Lucas, September 21, 2009, Cristina Burrola Aguilar CB08303 (MEXU 25752); Capilla Vieja, September 21, 2009, Cristina Burrola Aguilar CB08367 (MEXU 25768); Corral de piedra, September 21, 2009, Cristina Burrola Aguilar CB08331 (MEXU 25762); La Puerta, August 21, 2009, Roberto Garibay Orijel GO-2009-088 (MEXU 26104); Las Peñas, September 02, 2009, Cristina Burrola Aguilar AR09690 (MEXU 25874); Zinacantepec, Buenavista, August 12, 2008, Luis Antonio García Almaraz HC-PNNT-149 (MEXU 26635); Zinacantepec, El Contadero de Matamoros, September 03, 2008, Cristina Estrada Velazquez HC-PNNT-266 (MEXU 26701). Jalisco: Ciudad Guzmán, Km 15 del camino de ascenso al Nevado de Colima, no date, Castro-Castro 7 (IBUG); Nevado de Colima, camino a las antenas, October 24, 2010, Landeros 3401 (IBUG). Querétaro: Colón, Cerro El Zamorano, October 01, 2016, Landeros 3581 (IBUG).

Extralimit species specimens: Estado de México: Coatepec Harinas, July 23, 2008, Miguel Angel Pérez Villegas, HC-PNNT-059 (MEXU 26585); Tlalmanalco, Parque Ejidal, September 06, 2009, Roberto Garibay Orijel GO-2009-279 (MEXU 26201); Cerro Prieto, faldas del Nevado de Toluca, October 18, 2003, LGD 9002 (IBUG).

Comments: Nguyen *et al.* (2013) pointed that the geographical region and host association were the most important traits to distinguish species within the *H. lacunosa* species complex. *Helvella jocatoi* differentiates morphologically from *H. rugosa* and *H. sulcata* by its darker and lacunose stipe, bigger ascoma and association to *Abies religiosa*. It is different from *H. pseudolacunosa*, which has whitish or grayish stipe, developing in *Pinus koraiensis* and *Larix gmelinii* forests in China (Ariyawansa *et al.* 2015). While *H. dryophila* is associated to *Quercus* forests and *H. vespertina* to coniferous forest from western USA (Nguyen *et al.* 2013), *Helvella jocatoi* is endemic to central Mexico associated with *Abies religiosa* forests on the Trans Mexican Volcanic Belt. A resume of interspecific differences of closest species is shown in Table 2. All extralimital specimens from Mexico came from *Pinus* or mixed forest with *Quercus*, therefore they do not belong to *H. jocatoi*.

Discussion

So far the *H. lacunosa* species complex has 13 species distributed in Europe, Asia and North America (Figure 2B). The ITS-LSU concatenated phylogeny has strong support values both at terminal and deep branches. Our results corroborate those previously reported by Nguyen *et al.* (2013), Wang *et al.* (2013), Ariyawansa *et al.* (2015) and Skrede *et al.* (2017). However, the LSU alone does not has enough variation to separate all species. Since *H. palustris*, *H. philonotus* and the Neotype of *H. lacunosa* lack ITS sequences, the synonymy between *H. palustris* and *H. philonotus* and those between *H. lacunosa* and *H. pseudolaculosa* must be evaluated (Figure 2B). Additionally, there are still samples from China, Norway and Sweden labeled as "*H. lacunosa*" close to *H. rugosa*, which probably represent an unnamed species.

Helvella lacunosa s.l. was previously reported with a large distribution in Mexico (Landeros *et al.* 2013); however, DNA sequences from Mexican specimens in Nguyen *et al.* (2013) and the sequences generated in the present study indicate that they represent at least three unnamed species in this complex in Mexico. The most widespread and representative of them is described here as *H. jocatoi*. The description of additional diversity in the *H. lacunosa* from Mexico waits for more collections and host data associations.

Helvella jocatoi is described from the Trans Mexican Volcanic Belt that is a biogeographical province crossing central Mexico from East to West with unique biographical and ecological dynamics. Climatic fluctuations together with the volcanic activity generate a high animal and vegetal species diversification and local endemisms on each mountain from this province (Mastretta *et al.* 2015). From this region new mushrooms records and new mushrooms species have been reported recently (Piña-Páez *et al.* 2017, Contreras-Pacheco *et al.* 2018) suggesting that the Trans Mexican Volcanic Belt is also an important diversification center for ectomycorrhizal mushrooms.

The recognition of *H. jocatoi* as an independent taxon different from *H. lacunosa* s.s has implications for its use and conservation. The *Abies religiosa* habitat of *H. jocatoi* is endangered since 55% of its land cover disappeared in the last 40 year (Ramirez *et al.* 2015). In climatic change models, *Abies religiosa* forests will contract its current land cover in 70% by 2030 (Sáenz-Romero *et al.* 2012). Also, *H. jocatoi* and the two unnamed species in the *H. lacunosa* species complex are edible and have high cultural importance (Figure 4) (Burrola *et al.* 2012) and large sale volume in local and regional markets (Pérez-Moreno *et al.* 2008, Lara-Vázquez *et al.* 2013). In consequence, the climate change threatens the habitat of *H. jocatoi* and the large extraction volumes for sale could affect this and other related species. Finally, more studies are required to understand the effect of habitat reduction and mushroom harvesting in these edible species populations and to establish policies for species conservation.

FIGURE 4. *Helvella jocatoi* and related species sale in a rural market in central Mexico.

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