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Elaphomyces readii (Elaphomycetaceae, Eurotiomycetes), a new medicinal species of hypogeous fungus with biocultural importance from Mexico

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Abstract

Elaphomyces readii is presented as a new species to science based on morphological and molecular evidence. The new species is characterized mainly by the pale brown rugose ascomata, yellowish to reddish brown hues in the proximity to the gleba, dark purple gleba and ascospores 20–57 µm in diameter. *Elaphomyces readii* is morphologically similar to *E. papillatus* and *E. muricatus* but differs in the ascospore size and phylogenetical location. Further, *E. readii* is sold in traditional markets in the state of Oaxaca, southeastern Mexico as a medicinal fungus consumed to heal wounds, internal bleeding, and external injuries. Morphological descriptions, SEM ornamentation spores and nrITS-based phylogenetic analysis using maximum parsimony, maximum likelihood method and Bayesian inference support the proposal of this new species.

Keywords: Ectomycorrhizal fungi, Elaphomycetaceae, ethnomycology, sequestrate fungi, truffle-like fungi

Introduction

Elaphomyces T. Ness, also known as deer truffles, is a genus within Elaphomycetaceae (Eurotiales, Ascomycota) characterized by the hypogeous to subhypogeous ascomata, showing a wide variety of colors and generally showing a mycelial layer covering a hard and thick peridium. Most of the *Elaphomyces* species show ornamented spores with spines and ridges. The genus is distributed worldwide and it forms ectomycorrhizal associations with several species of Angiosperms and Gymnosperms (Castellano *et al.* 1986; Pegler *et al.* 1993). The genus has been intensely studied in the northern hemisphere, especially in America and Europe since Vittadini (1831). Further, some species are believed to be medicinal since the XVIII century (Trappe *et al.* 1979; Paz *et al.* 2017). Nowadays some studies have revealed anti-inflammatory and antioxidant activities (Saltarelli *et al.* 2008; Wang & Marcone, 2011; Chauhan *et al.* 2021). Recently several new species have been described from tropical forest in Africa (Buyck *et al.* 2016; Castellano *et al.* 2016), Asia (Zhang *et al.* 1991; Shirakawa & Tanaka, 2020) and America (Castellano *et al.* 2012, 2018, 2021).

The first records of the genus *Elaphomyces* in Mexico were made by Heim (1957) when he recorded that *Elaphomyces* specimens, known as *tlalipantli* "the earth within earth" together with *Psilocybe muliercula* Singer & A.H. Sm. were sold in traditional markets and possibly used in ritual ceremonies. These authors also recorded its use in the state of Oaxaca where they were known as "itamo real" and used to treat serious injuries (Trappe *et al.* 1979). Taxonomic studies have subsequently been carried out in northern Mexico (Castellano *et al.* 2012) and the Neo volcanic axis (Gómez-Reyes *et al.* 2012; 2018). Currently, eight species have been recorded in the country, mainly from mixed, coniferous and cloud forests (García-Romero *et al.* 1970; Trappe & Guzmán, 1971; Trappe *et al.* 1979; Cazares *et al.* 1992; Castellano *et al.* 2012; Gómez-Reyes *et al.* 2012; 2018).

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During ethnomycological studies in the Mexican state of Oaxaca, the author for correspondence of the present research recorded the sale of a species of *Elaphomyces* in a traditional market used as a remedy to internal wounds and bleeding, similar to the observations made by Trappe *et al.* (1979). After a deeper study of the samples purchased from different vendors, the *Elaphomyces* species showed big ascospores reaching up to 57 µm wide, ornamented with small flat papillae, sometimes coalescing and forming a sub-reticulum. Due to its unique morphological and molecular characteristics obtained of the nrITS region the sampled taxon is proposed here as a new species. The discovering of this new species with biocultural importance in Mexico hopes to encourage new ethnomycological as well taxonomic studies on sequestrate fungi.

Material and methods

Field work

Ascomata were collected in a traditional market in the city of Tlaxiaco, Oaxaca, located in southwestern Mexico. The specimens were sold by Mixtec women which inhabit the surrounding communities where pine forests proliferate. Color description was according to Kornerup & Wanscher (1978). The specimens were curated and deposited in the herbarium of *Instituto Tecnológico de Ciudad Victoria* (ITCV). Handmade cuts were made on dried specimens and mounted using 3% KOH and Melzer. At least 30 ascospores and asci were measured, as well as peridium structures. SEM images were obtained in the Instituto de Biología from Universidad Nacional Autónoma de México.

DNA extraction, amplification, sequencing, and phylogenetic analyses

DNA of ascomata were extracted using Aidlab™ kit (Beijing). The Internal Transcribed Spacer (ITS) region was amplified with the primer ITS5-ITS4 (White *et al.* 1990). Each 25 µl PCR mixture consisted of 2.5 µL 10 × PCR buffer (Mg2+), 1.5 µL dNTPs (1 mM), 1 µL BSA (0.1%), 1 µL each primer (5 µM), 1 µL 25-fold diluted DNA extracts, 0.5 µL MgCl2 (25 mM), and 1.5 U Taq DNA polymerase (Takara, Takara Biotechnology, Dalian Co. Ltd, China) using ddH_2O. The amplifications were performed with the following cycling parameters: 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, and with a final extension at 72 °C for 10 min. The PCR products were verified by agarose gel electrophoresis run for 1 h at 95 V cm -3 in 1.5% agarose and 1× TAE buffer (Tris Acetate-EDTA). The PCR products were purified and sequenced forward and reverse sequences at TsingKe Biological Technology (Beijing, China) using ITS1F and ITS4R primers. Sequences were edited using GENEOUS PRIME and queried against the NCBI public database GenBank with the BLAST algorithm for identification 2.2.19 tool (Zhang *et al.* 2000).

In order to study phylogenetic relationships, our newly produced sequences of two individuals of the new species, were added to reference sequences of nrITS deposited in the NCBI database (http://www.ncbi.nlm.nih.gov/genbank/) (Table1). The nrITS dataset included sequences from 44 specimens representing 20 taxa. The nrITS region was aligned using the online version of MAFFT v. 7 (Katoh *et al.* 2019; Katoh & Standley, 2013). The alignment was revised in PhyDE v. 10.0 (Müller *et al.* 2005), followed by minor manual adjustments to ensure character homology between taxa. The matrix was composed of 44 individuals (750 characters). The data were analyzed using maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). Maximum parsimony analyses were carried out in PAUP* 4.0b10 (Swofford, 2002) using the heuristic search mode, 1000 random starting replicates, and TBR branch swapping, with MULTREES and Collapse on. Bootstrap values were estimated using 1000 bootstrap replicates under the heuristic search mode, each with 100 random starting replicates. Maximum likelihood analyses were carried out in RAxML v. 8.2.10 (Stamatakis, 2014) with a GTR + G model of nucleotide substitution. To assess branch support, 10000 rapid bootstrap replicates were run with the GTRGAMMA model. Bayesian inference was carried out in MrBayes v. 3.2.6 x64 (Huelsenbeck & Ronquist, 2001) with four chains and the best evolutionary model for alignment was sought using PartitionFinder (Frandsen *et al.* 2015; Lanfear *et al.* 2014; 2017), the best-fit model selected for these three partitions of nrITS sequences was GTR+G for ITS1, JC for 5.8s, and HKY+G for ITS2. The information block for the matrix includes two simultaneous runs, four Montecarlo chains, temperature set to 0.2 and sampling 10 million generations (standard deviation ≤0.1) with trees sampled every 1000 generations. The first 25% of samples were discarded as burn-in, and convergence was evaluated by examining the standard deviation of split frequencies among runs and by plotting the log-likelihood values from each run using Tracer v. 1 (Rambaut *et al.* 2018). The remaining trees were used to calculate a 50% majority-rule consensus topology and posterior probabilities (PP). Trees were visualized and optimized in FigTree v. 1.4.4 (Rambaut, 2018).

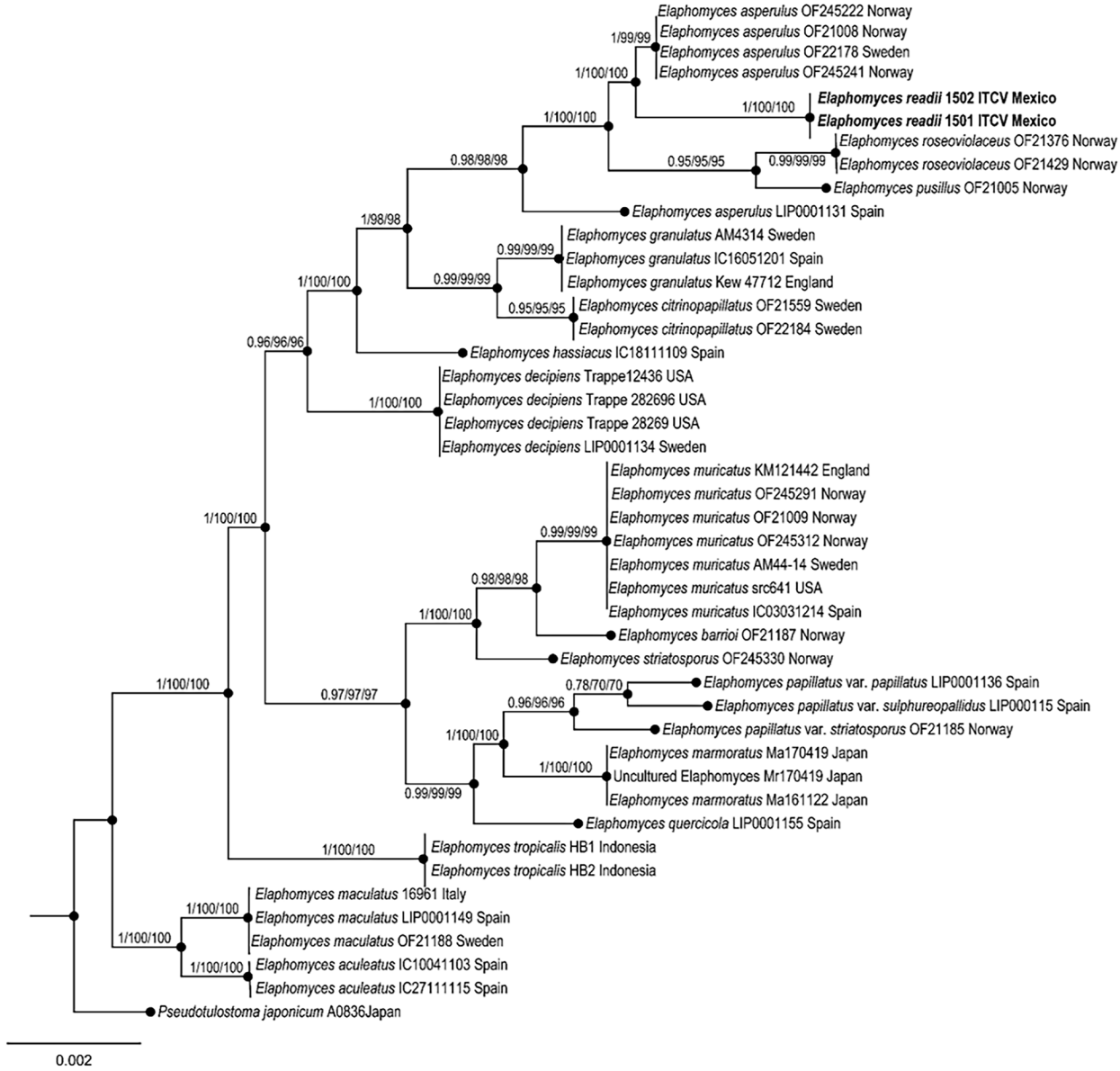

FIGURE 1. Maximum likelihood phylogeny based on the nrITS sequences data. Maximum parsimony and Bayesian analyses recovered identical topologies with respect to the relationships among the main clades of the *Elaphomyces*. For each node, the following values are provided: posterior confidence (p-value) / maximum parsimony bootstrap (%) and maximum likelihood bootstrap (%). The scale bar represents the expected number of nucleotide substitutions per site. The new species *Elaphomyces* section *Elaphomyces* is shown in bold.

Results

Molecular analyses

We successfully amplified and sequenced the nrITS region from two specimens of *Elaphomyces readii*. After incorporation of additional sequences downloaded from GenBank (Table 1), the aligned nrITS dataset included 805 characters (including gaps), of which 214 sites were conservated, 580 were variable sites and 482 were parsimony-informative. The three phylogenetic analyses, MP, ML, and BI, of the nrITS dataset recovered similar topologies (Fig. 1). No significant conflict (bootstrap value >80%) was detected among the topologies obtained via the separate phylogenetic analyses. The parsimony analysis of the alignment found 845 trees of 210 steps (CI=0.4017, HI=0.1874, RI=0.4325, RC=0.3258). The best RAxML tree with a final likelihood value of –32585.924927 is presented. The matrix

had 1041 distinct alignment patterns, with 4.47% undetermined characters or gaps. Estimated base frequencies were as follows: A= 0.115874, C= 0.287452, G= 0.145210, T= 0.158961; substitution rates AC= 1.015425, AG= 1.158745, AT= 1.025842, CG= 1.004752, CT= 4.147852, GT= 1.100000; gamma distribution shape parameter α= 0.001258. In the Bayesian analysis, the standard deviation between the chains stabilized at 0.005 after 3 million generations. No significant changes in tree topology trace or cumulative split frequencies of selected nodes were observed after about 0.25 million generations, which were discarded as 25% burn-in. In the present study, a new species based on morphological characters and phylogenetic analysis of nrITS (GenBank accession numbers OM569489 and OM569490) sequences is described (Fig. 2). The analysis of nrITS produced a phylogenetic tree where *E. readii* is shown as a monophyletic group (BS = 100%, BS = 100%, BI p = 1), together to *E. asperulus* but in a different branch.

TABLE 1. Species, vouchers, countries and corresponding GenBank accessions of the *Elaphomyces* specimens used for the phylogenetic analysis.

Taxon	Voucher	Country	ITS	References
Elaphomyces aculeatus	IC27111115	Spain	KX238821	Paz *et al.* 2017
E. aculeatus	IC10041103	Spain	KX238844	Paz *et al.* 2017
E. asperulus	OF22178	Sweden	KR029755	Shirakawa & Tanaka, 2020
E. asperulus	OF245241	Norway	KX165347	Shirakawa & Tanaka, 2020
E. asperulus	OF245222	Norway	KX165351	Shirakawa & Tanaka, 2020
E. asperulus	O-F21008	Norway	KX238791	Paz *et al.* 2017
E. asperulus	LIP-0001131	Spain	KX238833	Paz *et al.* 2017
E. barrioi	OF21187	Norway	KR029745	Shirakawa & Tanaka, 2020
E. citrinopapillatus	OF22184	Sweden	KR029762	Shirakawa & Tanaka, 2020
E. citrinopapillatus	OF21559	Sweden	KR029765	Shirakawa & Tanaka, 2020
E. decipiens	Trappe 12436	USA	EU837229	Shirakawa & Tanaka, 2020
E. decipiens	Trappe 28269	USA	EU846311	Shirakawa & Tanaka, 2020
E. decipiens	OF21484	Sweden	KR029742	Shirakawa & Tanaka, 2020
E. decipiens	LIP-0001134	Spain	KX238832	Paz *et al.* 2017
E. granulatus	Kew K(M)47712	England	EU784197	Brock *et al.* 2009
E. granulatus	AM4314	Sweden	KR029767	Molia *et al.* 2020
E. granulatus	IC16051201	Spain	KX238835	Paz *et al.* 2017
E. hassiacus	IC18111109	Spain	KX238834	Paz *et al.* 2017
E. maculatus	16961	Italy	JF907988	Osmundson *et al.* 2013
E. maculatus	OF21188	Sweden	KR029775	Molia *et al.* 2020
E. maculatus	LIP-0001149	Spain	KX238799	Paz *et al.* 2017
E. marmoratus	M-a161122	Japan	LC500962	Shirakawa & Tanaka, 2020
E. marmoratus	M-a170419	Japan	LC500963	Shirakawa & Tanaka, 2020
E. muricatus	src641	USA	DQ974740	Smith *et al.* 2017
E. muricatus	K(M)121442	England	EU784198	Brock *et al.* 2009
E. muricatus	AM44-14	Sweden	KR029730	Molia *et al.* 2020
E. muricatus	OF245291	Norway	KR029733	Molia *et al.* 2020
E. muricatus	OF245312	Norway	KR029736	Molia *et al.* 2020
E. muricatus	OF21009	Norway	KR029739	Molia *et al.* 2020
E. muricatus	IC03031214	Spain	KX238843	Paz *et al.* 2017
E. striatosporus	OF245330	Norway	KR029748	Molia *et al.* 2020
E. papillatus var. papillatus	LIP-0001136	Spain	KX238819	Paz *et al.* 2017
E. papillatus var. striatosporus	O-F21185	Norway	KX238790	Paz *et al.* 2017
E. papillatus var. sulphureopallidus	LIP-0001156	Spain	KX238830	Paz *et al.* 2017

.....continued on the next page

TABLE 1. (Continued)

Taxon	Voucher	Country	ITS	References
E. pusillus	OF21005	Norway	KR029757	Molia *et al.* 2020
E. quercicola	LIP-0001155	Spain	KX238837	Paz *et al.* 2017
E. readii	**1501-ITCV**	**Mexico**	**OM569489**	**This study**
E. readii	**1502-ITCV**	**Mexico**	**OM569490**	**This study**
E. roseoviolaceus	OF21429	Norway	KR029751	Molia *et al.* 2020
E. roseoviolaceus	OF21376	Norway	KR029752	Molia *et al.* 2020
E. tropicalis	HB1	Indonesia	LC010285	Sukarno *et al.* 2014
E. tropicalis	HB2	Indonesia	LC010286	Sukarno *et al.* 2014
Pseudotulostoma japonicum	A0836/TNS/KHJPN09408	Japan	KU934217	Buyck *et al.* 2016
Uncultured Elaphomyces	M-r170419	Japan	LC500964	Shirakawa & Tanaka, 2020

Taxonomy

Elaphomyces readii Pérez-Moreno & de la Fuente, *sp. nov.* (Fig. 2).
Mycobank no. 846810

Diagnosis:—*Elaphomyces readii* is mainly characterized by the peridium yellowish to reddish brown in the outer part, then whitish, with yellowish to reddish brown hues in the proximity to the gleba and ascospores ranging 20–57 µm in diameter, ornamented with small flat papillae, sometimes coalescing forming a subreticulum The macro- and micromorphological characteristics in conjunction with the nrITS-based phylogenetic analysis support the proposal of this new species.

Holotype:—MEXICO. Oaxaca: Tlaxiaco City, state of Oaxaca, purchased in a traditional market, 17° 07' 17. 21" N, 97° 34' 97. 50" W, 2040 m a.s.l., 17 July 2016, Pérez-Moreno J. (2501-ITCV, holotype).

Etymology:—This species is dedicated, as "*readii",* in honor to Professor Sir David J. Read, eminent mycologist, Emeritus Professor of the University of Sheffield, England, Secretary of the Royal Society (London) and a giant scientist in the study of the mycorrhizal symbioses (like the giant spores of the new species, averaging 52.33 µm in diameter), with more than 55 years of experience contributing to the understanding of the ecophysiology of mycorrhizal symbioses, and was knighted in 2007 by the Queen of England for his outstanding scientific contributions.

Description:—*Ascomata* 20–45 mm, globose to sub globose, slightly flattened when mature, sometimes lobulate, yellowish to reddish brown (5F4–5E4), dry, finely ornamented with flat papillae, crowded (2–3 papillae per mm), without mycelial strands. *Peridium* up to 1 to 1.5 mm thick, yellowish in the outer part, then whitish, yellowish to reddish brown near the gleba, firm, hard when dried, sometimes bruising reddish or orange when cut. *Gleba* pulverulent, white, then purple (18F8), dark purple (18F6) to blackish when mature, with some pale fibrils radially arranged, odor and taste fungoid. *Peridium* composed of three layers. *Exoperidium* 100–230 µm thick, composed of prostrate and horizontally arranged hyphae, 4–7 µm in diameter, hyaline or with golden content, septate, thin-walled, forming irregular pyramids of 400–650 µm. *Mesoperidium* 800–1200 µm thick, composed of interwoven tubulous hyphae, compacted, 3-5 µm in diameter, thin-walled. *Endoperidium* 250–450 µm thick, similar in structure to the mesoperidium, with tubulous interwoven hyphae, 5–15 µm in diameter, thick-walled, some with yellowish to reddish brown incrustations in the cell walls. *Asci* 45–62 × 38–42 µm, ellipsoid, rarely globose, hyaline, 6–8 spored, some with a pedicel up to 7 µm long, thick walled. *Ascospores* 20–57 µm (Average=52.33 µm, n=50), globose, with slender spines, crowded, reaching 3-5 µm long, sometimes converging in small ridges, forming a sub-reticulum or alveoli up to 5 µm in diameter, brown in 3% KOH, some with a big guttula, thick-walled.

FIGURE 2. *Elaphomyces readii*. **A.** Fresh ascomata in different stages (Holotype). **B.** Large collection from one market seller. **C.** Mixtec woman selling fresh ascomata (right) and red corals from the Pacific Ocean (left) with medicinal purposes in a Mexican traditional market. **D**. Exoperidium. **E**. Mesoperidium. **F**. Endoperidium. **G**. Mature ascospores. **H**. Asci. **I**. Detail of the ascospore ornamentation under SEM. Scale bars: **A and B**= 20 mm; **D**= 20 µm; **E-H**=10 µm.

Habitat, habit, distribution:—Solitary to disperse. So far only known from the type community in Tlaxiaco municipality, growing under *Pinus* spp. from the beginning of July to the end of August, having a short distribution in the middle of the rainy season phenology.

Specimens examined:—MEXICO. Oaxaca: Tlaxiaco Municipality, purchased in a traditional market, 17° 07' 17. 21'' N, 97° 34' 97. 50'' W, 2040 m, 20 August 2016, Pérez-Moreno J. (2501, 2502, 2503, 2504, 2505 ITCV).

Discussion

The main characteristics of the new species are the combination of pale brown rugose ascomata, with brownish to yellowish hues in the peridium proximity to the gleba, big ascospore size averaging 52.33 µm in diameter and ornamentation spore composed by slender spines, reaching up to 3–5 µm long, converging in small ridges, forming a subreticulum or alveoli. Morphologically, the ascoma is similar to *E. papillatus* Vittad. but that European species has smaller ascospores ranging 11–19 µm in diameter and non-alveolate ornamentation (Paz *et al.* 2017). It is also similar to *E. muricatus* Fr. from Mexico (Gómez-Reyes *et al.* 2012) but that species has smaller ascospores (up to 36 µm) and paler gleba when immature. *Elaphomyces verruculosus* Castellano also has big ascospores up to 45 µm in diameter, nevertheless, that species has a reddish epicutis and white gleba when young (Castellano *et al.* 2012). Due to the papillate and brownish hues, as well as the ascospores ornamented with a thick layer of spines/hairs the new species is located within the section *Elaphomyces*, subsection *Elaphomyces* according to Paz *et al.* (2017). The ITS phylogeny places the new species also closely related to *E. roseoviolaceus* A. Molia & E. Larss. and *E. pusillus* A. Molia & S. Sivertsen. Both species share the dark purple gleba and yellowish brown peridium with flat papilla, nevertheless, they can be separated from *E. readii* due to the ascospore size and geographical distribution (Jeppson *et al.* 2020; Molia *et al.* 2020). The new species, *E. readii*, resembles also the European species *E. asperulus* Vittad. in gleba color but it can be easily separated by the characteristic purplish-tinged peridium, ascospores ornamented by patches of confluent warts (Paz *et al.* 2017) and ascospore size (22-30 µm) in the latter species. *Elaphomyces readii* spore size is much bigger, with an average diameter of 52.33 µm, in mature specimens and a well-differentiated spore ornamentation. Phylogenetically related species to *E. readii* and diagnostic distinctive characters are shown in Table 2.

TABLE 2 Comparative morphology of diagnostic characteristics of related species to *Elaphomyces readii* within *Elaphomyces* sect. *Elaphomyces* according to Paz *et al.* 2017[1] and Molia *et al.* 2020[2]

Species	***E. readii***	***E. asperulus*[1]**	***E. pusilus*[2]**	***E. roseoviolaceus*[2]**
Ascospores (µm)	20–57	22–30	20–33	26–34
Asci (µm)	45–62 × 38–42	32–38 × 35–42	not measured	not measured
Distribution	North America	Europe	North Europe	North Europe
Ascoma size (mm)	20–45	20-40	4–18	13–25
Gleba color	whitish, purple, then dark purple	purple, then black purple	Greyish black, then violaceous to brownish black	black to violaceous
Plant hosts	*Pinus*	*Corylus, Castanea, Picea, Quercus, Pinus*	*Picea*	*Picea* and *Pinus*

According to all of the market sellers, who were the actual gatherers of the studied specimens the studied ascomata were collected under *Pinus* spp. This is an abundant type of vegetation in the surrounding area where the new species was bought. *E. readii* is one of the southernmost records of the genus in Mexico. As mentioned above, *E. readii* is sold in traditional markets as a cure for internal wounds. Similar information was observed by Trappe & Guzmán (1971) and Trappe *et al.* (1979) in the state of Mexico, Puebla and Oaxaca. The species studied by those authors were identified as *E. muricatus* and *E. reticulatus* Vittad.

Elaphomyces readii is commonly sold in the traditional market of Tlaxiaco, a city where Mixtec people sell a great variety of edible and medicinal products. These include ocean corals, medicinal plants, edible fruits and vegetables, and wild edible mushrooms included in the genera *Cantharellus, Ramaria, Laccaria* and *Tricholoma* gathered from the surrounding forests. *E. readii* is frequently known as "*itamo real*" (*ita* meaning flower in Mixtec language and *real* meaning "royal" in Spanish) or "*hongos de venado*" (or fungi of the deer) and it was recorded in the traditional market

in more than 15 stands, being sold mainly by women, who were the actual gatherers. All of the sellers commercialize this species because of its medicinal properties, since it helps in dealing with wounds, different types of inner bleedings, and it has anti-inflammatory properties. Therefore, it is locally used in a multitude of treatments including gastric or mouth ulcers, or any kind or sores. Sellers also mentioned that it is common among Mixtec people to give these fungal species to mothers who have recently given birth, seeking to aid in a speedy recovery. It is crucial for them to get back to their normal activities as soon as possible because of the marginal socioeconomic conditions they face. Additionally, *E. readii* currently constitutes a noteworthy part of Mixtec folklore. When asked how they discovered that this species possess the described medicinal properties, the sellers answered with a traditional belief: "… *there was a time when our ancestors used to hunt deer because they were very abundant and they liked the taste of their meat. But sometimes, when they only managed to injure the deer instead of killing them, they observed that the deer ran to some places where they knew these fungi grew; the deer dug around until they found the fungi and then ate them. Our ancestors saw that after some time the deer´s injures started to heal until their health was restored, so they tried to see if something similar happened to them when they ate the fungi themselves* …". It was interesting to hear the same story from different people belonging to different communities close to Tlaxiaco. Other people also shared stories related with their own experiences or experiences belonging to their close relatives being cured by the consumption of these fungi. Due to the fact that the Mixtec culture is ancient and they have been in contact with nature for the last three millennia at least, the formal research of anti-inflammatory, anti-hemostatic and antimicrobial bioactive compounds in *E. readii*, which have frequently been recorded in other ectomycorrhizal fungi (e.g., Pérez-Moreno and Martínez-Reyes, 2007) would be of great further interest.

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