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TAXONOMIC AND MOLECULAR CHARACTERIZATION OF FUNGI FROM SUGARCANE BAGASSE

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ABSTRACT

Agroindustrial waste represents an important source of energy for various species of fungi that can establish themselves in waste and use it as a substrate. In this study, a macrofungus that grows in the wild on sugarcane bagasse produced by the paper industry was taxonomically identified. Ten samplings or mushroom collections were made at the site, and an average of 6 individuals were collected per sampling. Specimens had a pileus between 4 and 15 cm in diameter, from parabolic to convex in shape, slightly umbilicated with soft odor and fibrous texture, brown on the surface and paler towards the margin, with tight lamellae of various lengths, ventricles, free union to stipe, and brown and pink in color. These characteristics allowed the identification of the specimens within the *Volvariella* genus. Molecular analyses using DNA sequencing of the basidia of the fungus allowed making comparisons with other *Volvariella* sequences stored in GenBank. A tree was generated by the maximum-likelihood method using the nucleotide substitution model HKY + I + G with 2.815 as the alpha parameter of the gamma distribution and 0.2990 as the proportion of invariant sites. The nucleotide frequencies were T = 0.2812, C = 0.2741, A = 0.2076, and G = 0.2372, while the phylogenetic reconstruction identified the species as *Volvariella volvacea*, with a 100% bootstrap. This is the first report of *V. volvacea* in southwestern Colombia and highlights the importance of the ligninolytic potential of this species as a food source and bioremediating agent.

Keywords: *Volvariella volvacea*, sugar cane bagasse, ligninolytic potential.

INTRODUCTION

From an ecological viewpoint, fungi are one of the most important groups of organisms on earth. They inhabit all ecosystems and are responsible for much of the decomposition of organic matter, increasing its availability in the soil (Nicolás et al., 2019; Fukasawa, 2021). Recent global estimates suggest that there are as many as 5.1 million fungal species; of which only 70,000 have been adequately described (Blackwell, 2011). According to Charria-Giron, fungi are found primarily in humid tropical forest ecosystems, such as the tropical dry forest (Charria-Girón et al., 2023), and oak forests present in very humid low montane forests. However, very few studies have examined the microbiota in other types of ecosystems such as the tropical dry forest (Holdridge, 2000), in which the presence of the Agaricales order of fungi has been reported, with an approximate inventory of 270 species (García Lemos and Bolaños Rojas, 2011).

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Considering their nutritional preference, fungi can be divided into: i) saprobes, when they decompose organic waste to feed themselves; ii) parasites, when they extract the organic substances they need from a host; and iii) symbiotic, when their association with the host allows them to survive. In the latter, there are many mutualist fungi capable of carrying micellar associates with insects, mycorrhizae, lichens, and endophytes (Chen et al., 2019). Through these symbioses, fungi allow a diversity of other organisms to exploit new habitats and resources. Macromycete fungi have attracted great interest in various fields because some species are phytopathogenic, resulting in economic losses. However, the vast majority of species have been positively recognized as sources of high nutritional value due to their pharmaceutical and mycorrhizal properties, as well as their great potential for biodegradation (Tripathi et al., 2017) .

Fungi grow in different environments, including the ground, pieces of dead wood, and decaying plants. These fungal organisms are important because they are related to the decomposition process (Fukasawa, 2021; Fukasawa and Matsukura, 2021). In the present study, a fungus was found growing on sugarcane bagasse waste from the paper industry in southwestern Colombia. Its characteristics suggested that it could be the edible fungus *Volvariella volvacea* (*V. volvacea*), but there was no previous record on the presence of this species in the wild in that habitat in the country. Therefore, this research aims to conduct a morphological and molecular identification of a fungal species growing on sugarcane bagasse produced by the paper industry in Colombia.

MATERIALS AND METHODS

The sampling area was located in Guachene, Cauca Department, Colombia (3° 11›34.13 «N-76 ° 24›13.75» W). The area has an average height of 1,050 m.a.s.l. and an average temperature of 25 ° C, with precipitation ranging from 1,000 to 1,200 cm^3 per year, and bimodal seasonality. It belongs to the tropical dry forest ecosystem according to the life zone classification of Holdridge (Holdridge, 1967).

Sampling of biological material: 10 samplings were carried out inside a paper company, where 60 carpophores were obtained. Climate variability due to the influence of El Niño affected the development of the species in the study area, decreasing the frequency of mushroom production during periods of parched weather.

The collected individuals were found in the courtyards of the company, at the base of the sugarcane bagasse open-pit hills, between 0 and 1.5 m high. Sampling was carried out in an approximate area of 7,000 square meters, in the morning hours to guarantee that morphological analyses could be carried out during the day. Most specimens were found growing preferably in the vicinity to small tributaries.

Photographs of the individuals were taken in situ, recording their fresh ecological and morphological characteristics. Subsequently, the samples were stored in paraffin-coated paper bags and transported to the biology laboratories of the Universidad del Valle, Cali, Colombia. Preservation was performed at 4 °C until analysis.

Isolation and morphological characterization. Macroscopic and morphological analysis

The isolation, characterization of the pile, hymenophore, stipe, morphology, and morphometry of the collected individuals was carried out. Photographs were taken and the internal structures of the individuals were observed using a stereoscopic microscope (NIKON, model SMZ 445). Queries were made online using taxonomic keys (Menolli, 2008; Shaffer, 1962).

Microscopic and morphological analysis

At the microscopic level, characteristic elements of the genus volvariella , piscipellis, lamella, cheilocistidios, and basidiospores were observed under a microscope (OLYMPUS, model CX21FS1) using the 100x and 400x magnifications. Taxonomic keys were run (Menolli, 2008; Seok et al., 2002; Shaffer, 1957) to identify species based on morphological characteristics.

The fungal structures were stained with Melzer reagent, which was prepared using the following formula: 22 mL distilled water, 20 g chloral hydrate, 0.5 g iodide, and 1.5 g potassium. A mixture of these ingredients was prepared for later use. Subsequently, the specimens were oven-dried at 50 ° C and finally deposited in the fungus collection of the Luis Sigifredo Espinal Tascon Herbarium (CUVC) of the Universidad del Valle.

For isolation of fungal strains, some individuals were washed with 2% hypochlorite, rinsed with 70% ethanol solution, and later with abundant distilled water, thus ensuring the elimination of other organisms that competed in growth in the culture medium.

For seeding, internal tissue samples were collected by making cuts with a sterile scalpel between the base of the pileus and stipe. The extracted samples were seeded in sterile culture media of Agar- malt extract + calcium carbonate and Potato Dextrose Agar (PDA) and incubated

at 34 ° C. After 5 days, it was possible to observe the growth of mycelium, which was isolated in progressive repetitions of culture until obtaining a pure culture.

Alternatively, strain isolation was performed from the spores of the collected individuals, multisporic, to which the spore was washed, sown on malt extract plus calcium carbonate agar, and incubated at 34 ° C. The germinated spore was selected and isolated repeatedly until a pure culture was obtained. The pure strain obtained from both isolates was stored as the mother strain in a refrigerator at -20 ° C.

DNA extraction and sequencing

Genomic DNA was extracted from fragments macerated in liquid nitrogen from the basidium of the fungus using a Power Soil DNA Isolation Kit (MOBIO LAB) according to the manufacturer's protocol. The extracted DNA was used to amplify the ribosomal region (ITS1-5.8SrDNA-ITS2) using the universal primers: ITS1 5 'TCCGTAGGTGAACCTGCGG 3) and ITS4 (5 TCCTCCGCTTATTGATATGC 3) according to the methodology used by Raju et al. (2014).

The PCR cocktail was prepared using the following final concentrations: buffer (1X) $MgCl_2$ (1.75 μM), dNTPs (5 μM each), primers (4 μM each), and Taq polymerase (1 unit). The program used for amplification consisted of a cycle of denaturation at 94 ° C for 5 min, followed by an amplification phase of 35 cycles, and denaturation at 94 ° C for 1 min. Hybridization was conducted at 54 ° C for 45 s and an extension at 72 ° C for 40 s, followed by a final extension phase of one cycle at 72 ° C for 10 min. The PCR product was verified and quantified using nanoDrop (A260nm, A280nm). Finally, the sequences were determined using standard procedures of the service provided by Macrogen (USA) (Darriba et al., 2012).

Identification and molecular classification

The sequences obtained were edited and compared manually with similar sequences deposited in the GenBank databases. Those that showed a greater relationship were collected, aligned with the Bioedit Sequence Alignment Editor program, and used in the construction of a maximum likelihood tree using the PAUP (phylogenetic analysis using parsimony) v.4.0b10 (Swofford, 2003). The nucleotide substitution model was selected using the Akaike criterion in ModelTest version 2.1.3 (Darriba et al., 2012), and statistical support was evaluated using the maximum likelihood bootstrap (ML) with 1,000 repetitions.

The phylogenetic history of the fungus collected was inferred from sequences of different species of the *Volvariella* genus; a tree was generated by the maximum likelihood method. The nucleotide substitution model HKY + I + G was used with 2.815 as the alpha parameter of the gamma distribution and 0.2990 as the proportion of invariant sites. The nucleotide frequencies were T = 0.2812, C = 0.2741, A = 0.2076, and G = 0.2372.

RESULTS AND DISCUSSION

Taxonomic identification and phylogenetic inference

Macroscopic morphology of the mushroom under study

Pileus: campanulate (bell-shaped), widely convex, and slightly umbonate in the center, with a soft smell and texture, white to very light brown in color, with yellow pigmentation in some fibrils towards the edge of the inferior margin. Diameters between 50 and 160 mm were compared with taxonomic keys (Fig. 1. A).

Hymenophore: tight, ventricular lamellae, and free union to the stipe. Presence of the cells; pink to light brown in color; spored pink-brown color (Fig. 1. B and C).

Stipe: 40 -160 mm in length x 5 and 15 mm in diameter. Insertion into the central pile, which is curved and has a width at the base and decreases in thickness towards the apex. Slightly bulbous, flexuous, fibrillated, and striated longitudinally towards the base. It has no ring (Fig. 1. D and E). It presents a volva of 20 and 45 mm in diameter, sacciform, darker in color than the stipe, broad, and well developed (Fig. 1. F).

Microscopic morphology of the mushroom

Volvareas characteristics: thin-walled hyphal cells

Pileipellis: composed of thin-walled hyphal cells arranged approximately parallelly (Fig. 2.A).

Plot of the lamella: bilateral convergent, formed by hyaline, septate, and thin-walled hyphae.

Cheilocistide: nailed, thin walled. Ninety-nine x 26 μm.

Pleurocystide: thin wall, appendiculate and ventricular, often with strangulation in the upper part. Forty x 5 μm.

Basidium: the structure appears to have a broad base with thin-walled, clavate and short sterigma (Fig. 2.B). The size (6.5 x 24 μm) matches the typical dimensions of a basidium, which is usually elongated and narrow.

Basidiospores: thin-walled, ellipsoid, smooth, guttulate and enameloid 5 - 10 μm. x 2.5 - 5, μm (Fig. 2.C).

According to Dutta et al. (2011) and Kumar

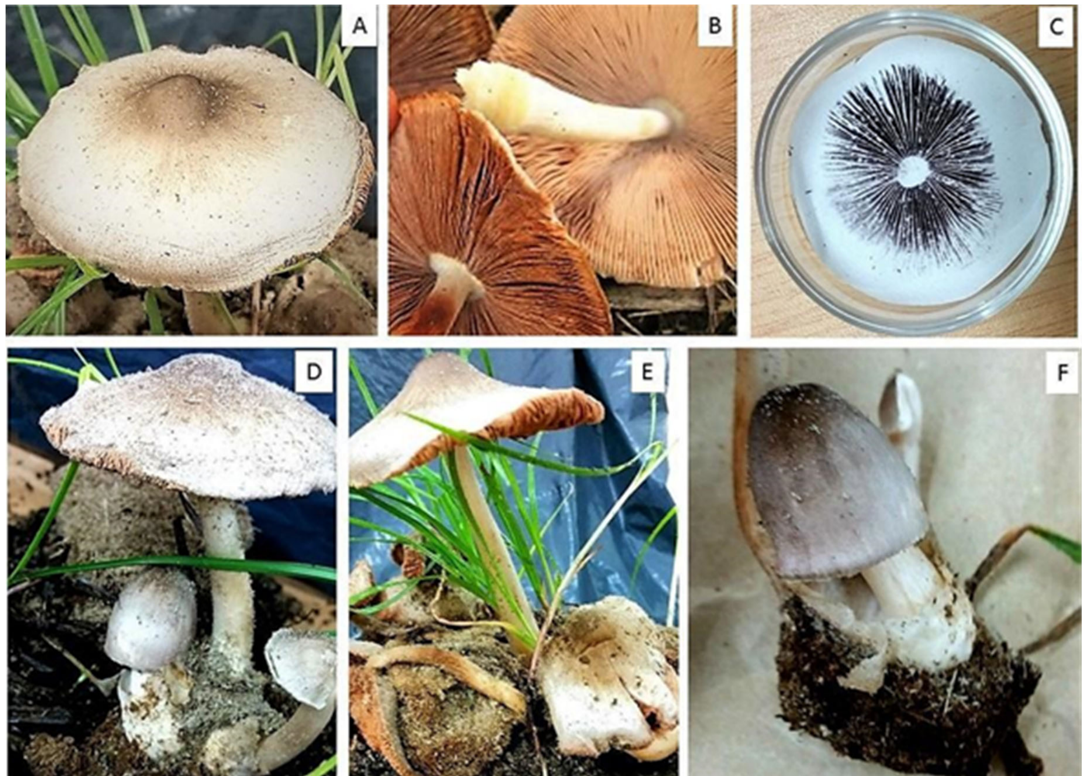

Fig. 1. Macroscopic morphology of the fungus found growing on a substrate of sugarcane bagasse in Guachene, Colombia.

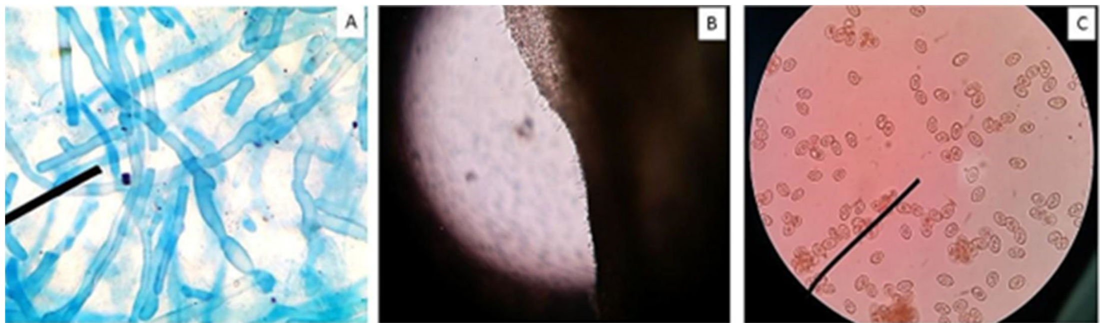

Fig. 2. Microscopic structures of the fungus growing on sugarcane bagasse. [A] and [B] seen at 400x; [C] view at 100x.

et al. (2022), the pileus of V. *volvacea* is 80 mm in diameter, parabolic to convex or hemispheric in shape on the surface. It is brown and paler toward the margin; it may have a smooth or fluted margin; the lamellae are composed of cells of various lengths and are pinkish brown. The stipe is central, slightly conical at the base, whitish in color, and lacks a ring. These characteristics correspond well with those previously described for the identified fungus (Fig. 1).

According to the macroscopic and microscopic characteristics of the mushroom and in agreement with the applied taxonomic keys, it can be presumed that it belongs to the genus *Volvariella*. Additionally, according to these characteristics, it is suggested that the fungus belongs to the *V. volvacea* species. However, molecular analyses were carried out to confirm its classification at the taxonomic level of the species. The sequence obtained from the ITS region was amplified,

giving 541 pairs of bases. Compared with the GenBank data bank, this amplicon shows an identity of 99.0% with *V. volvacea*.

Molecular identification and phylogenetic inference

DNA extraction: DNA was extracted according to the methodology described by Raju et al. (2014). For DNA analysis, five samples of the fungus were processed, all extracted from the same collection site. The results presented below correspond to one of these samples, since no significant differences were observed between the results obtained.

Sequencing: The sequences were determined using standard procedures of the service provided by Macrogen (USA). Manually edited sequences *Sequencher* 5.4.5 yielded 541 pairs of bases (Fig. 3); these were compared with the GenBank data bank showing 99.0% identity as *V. volvacea*.

Alignment of sequences with BLAST-N: The basic local alignment tool (Blast-N) available on the NCBI page related the sequences obtained specifically with the species: *V. volvacea* (Fig. 4).

Phylogenetic inference: Fig. 5 shows the tree made under the method of maximum verisimilitude, in which individuals of *V. volvacea* form clade A (H1 and H2 correspond to sequences obtained from the basidiome and H3 corresponds to the sequence obtained from mycelium isolated *in vitro*). This cluster is supported by a bootstrap value of 99%, indicating a high level of confidence in the clustering of *V. volvacea* sequences. Clade B corresponds to sequences of *V. bombycin*, another species within the genus *Volvariella*. Clades C and D correspond to sequences of other *Volvariella* species. In the basal zone of the tree, the genus *Pluteus* acts as a group to provide a reference point for evolutionary comparison.

The analysis shows that sequences H1, H2 and H3 coincide with the species *V. volvacea*, confirming the identification of the analyzed individuals as members of this species. Furthermore, the fact that this group was supported by a high bootstrap value (99%) reinforces the robustness of the classification.

The fungus identified as *V. volvacea* (Bull) Singer, 1951; belonging to the Family Pluteaceae, Agaricales Order, Agaricomycetes Class, Basidiomycota Phylum (Justo et al., 2011; Hibbett,

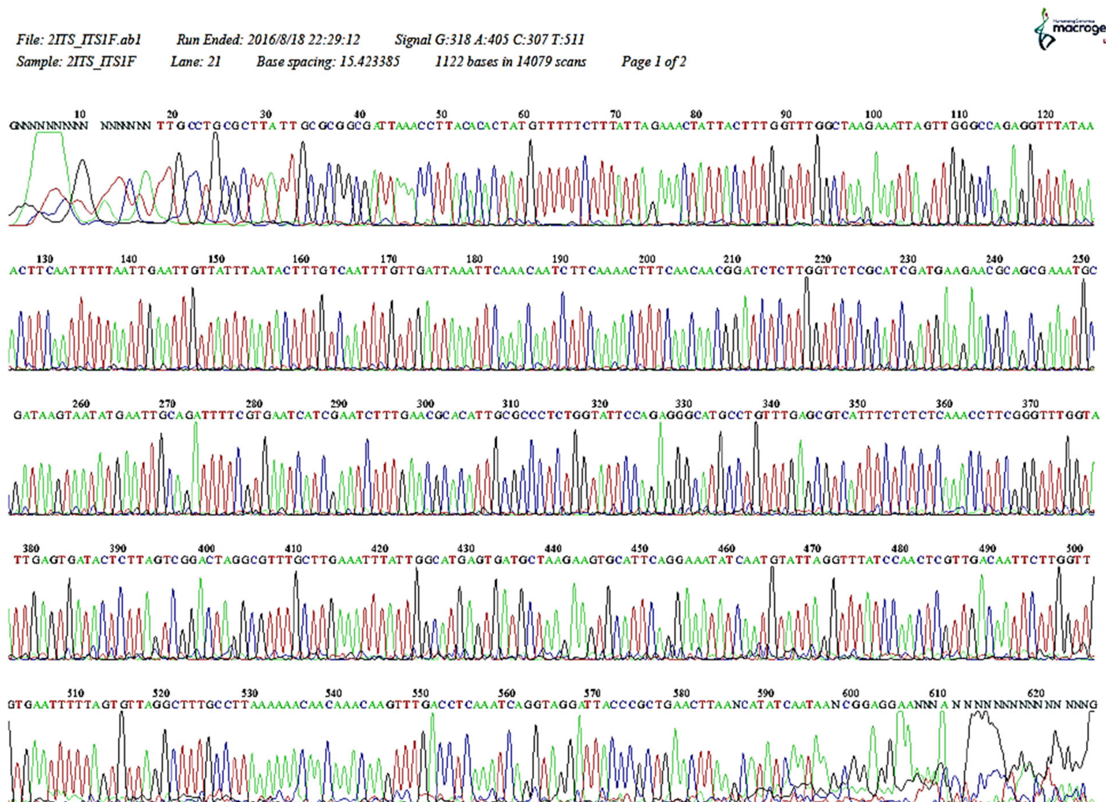

Fig. 3. Chromatogram obtained from sequencing the DNA sample of the analyzed mushroom.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Volvariella volvacea strain OE-12 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, coi	1328	1328	96%	0.0	99%	JN086667.1
Volvariella volvacea strain Vv-34 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, con	1327	1327	96%	0.0	99%	FJ379274.1
Volvariella volvacea strain V5-1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, comj	1325	1325	96%	0.0	99%	FJ379272.1
Volvariella volvacea strain OE-273 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, ci	1315	1315	94%	0.0	100%	KC142118.1
Volvariella volvacea strain 1206 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, com	1306	1306	94%	0.0	100%	JN086668.1
Volvariella volvacea strain OE-1222 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2,	1303	1303	94%	0.0	100%	JN086669.1
Volvariella volvacea strain V23 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, comp	1299	1299	95%	0.0	99%	FJ379273.1
Volvariella volvacea strain OSM-1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, co	1295	1295	93%	0.0	100%	KC142108.1
Volvariella volvacea strain OSM-3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S	1291	1291	93%	0.0	100%	KC142110.1
Volvariella volvacea strain OSM-2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, co	1291	1291	93%	0.0	100%	KC142109.1
Volvariella volvacea strain OSM-4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S	1286	1286	92%	0.0	100%	KC142111.1
Volvariella volvacea strain OE-272 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S	1279	1279	92%	0.0	100%	JN086662.1
Volvariella volvacea strain BBH-05 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S	1277	1277	92%	0.0	100%	JN086664.1
Volvariella volvacea clone 42 18S ribosomal RNA gene, partial sequence	1264	1264	91%	0.0	99%	KT120046.1
Volvariella volvacea strain OSM-6 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, co	1260	1260	93%	0.0	99%	KC142117.1
Volvariella volvacea strain OE-210-12 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2	1256	1256	96%	0.0	98%	JN086672.1
Volvariella volvacea strain ATCC MYA-4696 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed sp	1254	1254	93%	0.0	99%	HQ999973.1
Volvariella volvacea strain OSM-8 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S	1251	1251	92%	0.0	99%	KC142114.1

Fig. 4. Alignment generated using BLAST-N obtained specifically with the species: *Volvariella volvacea*.

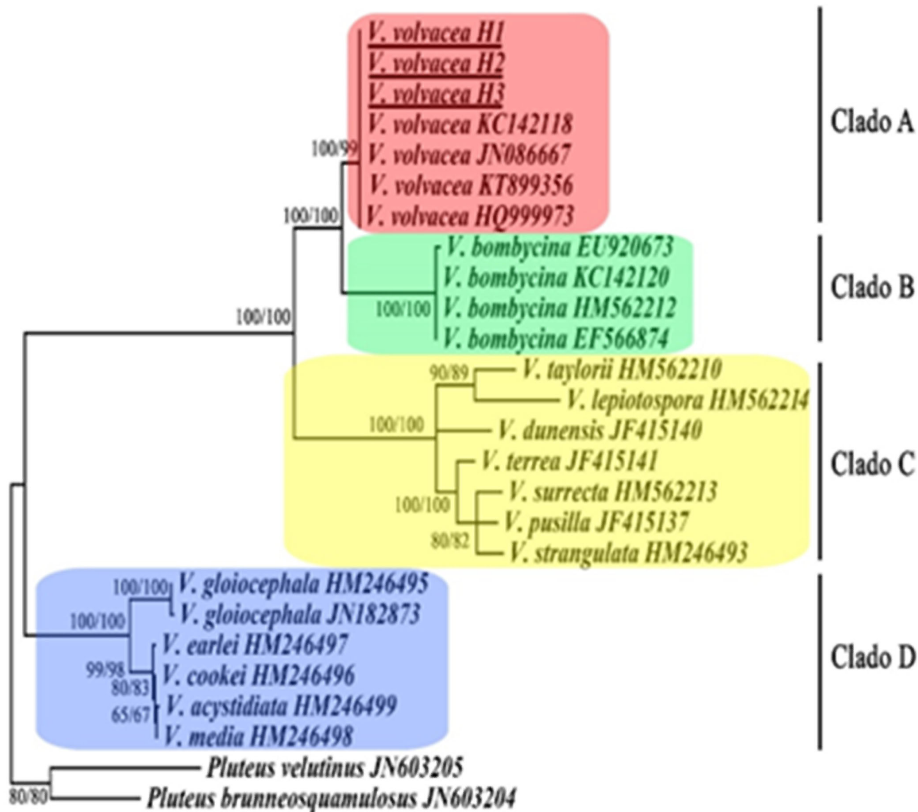

Fig. 5. Phylogenetic inference of the sequences of the genus *Volvariella*.

et al., 2007), is not yet reported in the regional distribution checklist of MYCOTAXON (Anon., sf) in Colombia. Until now, only the genera Amanita and Pluteus of the family Pluteaceae have been reported in the country (Vasco-Palacios and Franco-Molano, 2013), which means that this is the first report on the presence of a new genus of macromicetes in Colombia.

Ecological aspects of *Volvariella volvacea*

The fungus under study is a species of Chinese origin with saprophytic habit and gregarious growth. It grows on piles of rice straw, wood, and compostable waste. It is well distributed in tropical and subtropical zones, and its cultivation, which began during the 18th century in China, rapidly expanded throughout Southeast Asia due to its multiple nutritional benefits. In fact, fungi have been part of the human culture for thousands of years, being historically described as an important source of nutrition and health for different civilizations (Ahlawat and Tewari, n. d.; Li et al., 2021). Although some may be pathogenic, venous, or psychotropic, others are edible, saprophytic, or produce compounds with beneficial health properties, fulfilling antiviral, antibacterial, immunomodulatory, anticancer, antitumor, and hypocholesterolemic functions, among others (Chen et al., 2020; Li et al., 2021; Suárez Arango and Nieto, 2013; Yu et al., 2020).

Currently, this species is also distributed as a crop on the Iberian Peninsula, on the north and center of Europe, on the south coast and northwestern coast of the United States, and on the west of Mexico and Costa Rica. However, most research on this species has been mainly conducted in China and India (Ahlawat and Sagar, 2007; Ahlawat and Tewari, n.d.; Amir et al., 2023; Sánchez and Mata, 212 C.E.).

Cotton waste as a substrate for the cultivation of the fungus *V. volvacea* was introduced for the first time in Japan by 1971, completely replacing traditional rice straw by 1973. This was a turning point in the history of *V. volvacea* as its cultivation was conducted for the first time on an industrial scale in Hong Kong, being then implemented in Taiwan, Thailand, and China (Umor et al., 2020). After the 1980s, experimental cultures of *V. volvacea* with introduced strains were carried out in Mexico on substrates based on henequen bagasse, barley straw, coffee pulp, and banana crop waste. In addition, the growth of this species has also been reported in agroindustrial waste such as sugarcane bagasse. This makes *V. volvacea* particularly interesting because it is a possible solution to the environmental problem caused by the disproportionate production of wastes in the paper industry (Guzmán et al., 1993; Umor et al., 2020).

V. volvacea requires high temperature (35 ± 2 ºC) for optimal growth of the hypha. The fungus also needs 32 ± 2 ° C, and between 80 and 90% relative humidity for the formation of fruiting bodies, which takes about 10 days (Sakinah, 2019). These conditions are largely fulfilled in the area covered by the municipality of Guachene, where the *V. volvacea* fungus was found growing on piles of sugarcane bagasse close to small tributaries, which provided an adequate environment for fungal growth and development.

There are no previous records of *V. volvacea* in Colombia. In fact, its natural geographical distribution does not correspond to the American continent despite its widespread use in the United States and Mexico. It is plausible that this mushroom was a variety introduced by the company that discovered its presence. In this sense, imports of raw materials made by the paper company, which come mainly from the United States, Chile, Canada, Brazil, Mexico, Ecuador, Spain, China, Finland, and Germany (pulp, paper and graphic industry) may have facilitated the transit of latent spores of various organisms, whereby only those that find optimal environmental conditions for development can establish themselves.

As protein content in mushrooms is relatively high and comparable to that in eggs, milk and meat, *V. volvacea* constitutes an alternative source of protein. Additionally, given that *V. volvacea* is produced at high temperatures, it represents a potential productive alternative for the warm regions of the country, especially rural areas, since its cultivation requires low investment. Furthermore, *V. volvacea* can be used as a biocatalyst for the degradation of lignocellulosic material and lignin, which is abundantly found in agroindustrial waste to produce biofertilizers (Kumla et al., 2020).

CONCLUSIONS

Morphological and molecular data confirm the presence of *Volvariella volvacea* in sugarcane bagasse residues in Colombia. This study represents the first record of *V. volvacea* in southwestern Colombia, suggesting its potential adaptation to different lignocellulosic substrates in tropical regions.

The identification of *V. volvacea* represents a great contribution to the biodiversity records of the national mycobiota.

V. volvacea thrives in high temperatures and humidity, which makes it a viable option for the hot regions of Colombia. This is relevant because many rural areas of the country have these climatic conditions, which would facilitate

its production with a relatively low investment.

Limitations of the study

Currently, the use of a single molecular marker is not sufficient to ensure accurate species identification, suggesting the need for complementary studies using multiple genetic markers to increase confidence in phylogenetic results.

Author contribution

Conceptualization: T.K.M., Methodology: Carlos E. Villalobos, Marisol Gordillo, Adriana M. Chaurra., Validation: Carlos E. Villalobos, Marisol Gordillo, Adriana M. Chaurra. Investigation: Carlos E. Villalobos, Marisol Gordillo, Adriana M. Chaurra, and Diana P. Navia. Data analysis, Writing-original draft, Writing and editing: Carlos E. Villalobos, Marisol Gordillo and Adriana M. Chaurra. Supervision: Diana P. Navia. All co-authors reviewed the final version and approved the manuscript before submission.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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