New record of *Agaricus subrufescens* (Agaricales, Basidiomycota) for Ecuador

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**ABSTRACT**

*Agaricus subrufescens* (Agaricales, Basidiomycota), an edible species with attributed medicinal properties, has been reported from several countries of the world. This study presents the first record for Ecuador, based on morphological and molecular (ITS-5.8S of DNA) characterization for one wild specimen collected outside the Puyango Petrified Forest (PPF). This information marks the baseline for future research projects in areas such as the Puyango Petrified Forest, declared National Heritage of Ecuador, to valorise neotropical fungi with commercial potential.

**Keywords:** DNA; El Oro; ITS-5.8S; Loja; morphology.

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**RESUMEN**

*Agaricus subrufescens* (Agaricales, Basidiomycota), una especie comestible a la que se atribuyen propiedades medicinales, ha sido reportada en varios países del mundo. Este estudio presenta el primer registro para Ecuador, basado en caracterización morfológica y molecular (ITS-5.8S del DNA) de un espécimen silvestre colectado fuera del Bosque Petrificado Puyan-
INTRODUCTION

*Agaricus subrufescens* Peck (Agaricales, Basidiomycota) has been described in several countries of different continents, e.g. North and South America; Asia; Europe; and Oceania, and then considered cosmopolitan (Wasser et al., 2002; Kerrigan, 2005; Zhao et al., 2011; Thongklang et al., 2016; Zied et al., 2021). It is a saprotrophic species ecologically important for decomposition of organic matter, with reports of high nutritional and medicinal characteristics and commercial importance (Hetland et al., 2011). *Agaricus subrufescens* belongs to *Agaricus* section *Arvenses* (Cao et al., 2021), with notable variability in its macromorphology (robust or thin basidiomata) or very variable color on the surface of the pileus, presenting shades ranging from white to golden brown or violet brown, due to the genotype as influences of the climate and ecosystems where it develops (Kerrigan, 2005). Sometimes this species is difficult to distinguish morphologically from other species like *A. macrochlamys* (Medel-Ortiz et al., 2022).

Based on molecular information, *A. subrufescens* has been described from USA, and considered conspecific with some *Agaricus* species described from América, Europa and Africa: *A. brasiliensis* Fr. from Brazil; *A. blazei* Murrill from U.S.A.; *A. fiardii* Pegler from Martinique; *A. rufotegulis* Nauta from Netherlands; *A. bambusae* Beeli from Belgique (Wasser et al., 2002; Kerrigan, 2005; Thongklang et al., 2016; Ehrich & Hutter, 2020; Zied et al., 2021; Medel-Ortiz et al., 2022).

In South America, *A. subrufescens* has been reported from Brazil (Kerrigan, 2005), and mentioned in commercial production as *A. blazei* in Argentina (González et al., 2011) and in Ecuador Hidalgo & López (2019) report biological activity of a commercial strain *A. blazei*.

So far, no records of the species has been done in fungal diversity works for Ecuador, considered a megadiverse country [United Nations Environment Programme, 2014], (Cruz et al., 2016; Benítez et al., 2021; Gates et al., 2021; Toapanta-Alban et al., 2021). In addition, there are no data on the traditional use of this species by indigenous communities (Andrade et al., 2012; Gamboa-Trujillo et al., 2019). *Agaricus subrufescens* is commercially cultivated in several parts of the world due its nutraceutical importance (Mata & Savoie, 2012; Sánchez & Mata, 2012; Wisitrassameewong et al., 2012; Thongklang et al., 2016; Velázquez-Narváez et al., 2018; Zied et al., 2021).
The Petrified Forest of Puyango (PFP or Puyango Petrified Forest) in Ecuador, a protected ecosystem area, is recognized as National Heritage of Ecuador (Morante-Carballo et al., 2020). So far, no fungal species have been formally recorded in this area using a molecular approach (Læssøe & Petersen, 2008; Ordoñez, 2018; Batallas-Molina et al., 2021; Gates et al., 2021). However, only a preliminary checklist for PPF showed an interesting fungal diversity (Cruz & Masache, 2023).

In this work the species A. subrugescens is present as new record based in morphological and molecular data (ITS-5.8S DNA) for Ecuador.

MATERIALS AND METHODS

Material and studied area.— The specimen of Agaricus sp. was found in the outer limit of the PPF, and it was deposited in the mycoteca of the HUTPL Herbarium with the reference number HUTPL(F)2047. The PPF is located in southern region of Ecuador (Fig. 1).

Morphological analyses.— Macroscopic analyses were based on the observation (fresh sample) of the shape, texture and color of the basidiome. Color codes are based on the https online server https://encycolorpedia.es/a49074 (last viewed 28 March 2023). Macrochemical reaction on the pileus surface was tested with Potassium hydroxide KOH 10%. For microscopical analyses hand cuts of basidiome were made thought a Stemi Carl Zeiss stereomicroscope, and the preparations were stained with 1% Phloxine or 1%

![Fig. 1. Geographical Location of the Puyango Petrified Forest.](image-url)

Fig. 1. Ubicación Geográfica del Bosque Petrificado de Puyango.
Congo red and Melzer’s reagent. The samples were examined at 1000-fold (100X) magnification using an Olympus BX51 microscope.

The length and width of microstructures were taken from at least 30 measurements. The range of measurements includes averages (\(\bar{x}\)) of the most constant values and extreme values in parentheses. The Q-value of basidiospores (average spore length/average spore width) was recorded and basidiospore shape was classified according to Largent et al. (1973).

**DNA extraction and molecular analysis.**—DNA was extracted from the *Agaricus* sp., basidiome under the recommended protocol in the Phire Plant Direct Master Mix PCR Kit (Thermo Scientific™), followed by polymerase chain reaction (PCR) amplification using the unmodified primers ITS1 and NL4 (White et al., 1990). The final reaction volume was 20 μl including 1 μl target DNA for the reaction. PCR conditions were as follows: initial denaturation (98 °C, 5 min); followed by 40 cycles with denaturation (98 °C, 10 s); annealing (55 °C, 10 s); extension (72 °C, 30 s); and final extension (72 °C, 5 min). The PCR product was purified with Genomic Plant DNA Purification Kit (Invitrogen™ PureLink™). Sequencing (SANGER) was carried out at Macrogen INC (Seoul-Korea) using the same PCR primer pair.

The sequences were assembled and edited in Codon Code Aligner 5.1.4 (CodonCode Corporation, Centerville, MA, USA). All selected sequences (from basidiomata or strains) mainly correspond to the most similar (about 3%) and sequences named at species level with difference no more than 10% to sequences at the GenBank database (https://www.ncbi.nlm.nih.gov) as well as the UNITE database (https://unite.ut.ee) (Table 1).

Alignment of the obtained sequence and reference sequences was carried out in the MAFFT 7 program (Katoh et al., 2019) under the GINSI algorithm. Phylogenetically, Neighbour joining (NJ) analysis, Kimura 2-parameter (K2P) model with 1000 Bootstrap replicates, and Maximum Likelihood (ML), General Time Reversible (GTR) model with 1000 Bootstrap replicates, constructed in the MEGA X program (Stecher et al., 2020), were performed. The phylogenetic tree was outgroup rooted with sequences from *A. sodalis* and *A. megalosporus* belong to section Minores, and the values above the nodes correspond to ML and NJ respectively. The sequence of this study (GenBank accession number ON799184) is available in the National Center for Biotechnology Information (NCBI) database.
RESULTS

Molecular phylogenetic inference

Phylogenetically, 28 sequences were analyzed including our sequence from *Agaricus* sp. HUTPL(F)2047. The sequence of our specimen was clustered within *A. subrufescens* clade (Bootstrap value 99%) into the Arvenses section (Fig. 2). The *A. subrufescens* clade include sequences obtained from commercial and wild strains from several countries: *A. rufotegulis* culture from United Kingdom; *A. blazei* commercial and wild strains from Brazil BR; *A. subrufescens* wild specimen from Germany GER; commercial strains from China, North Korea; and a sequence obtained from a basidiome identified as *A. fiardii* voucher F2285 LIP Martinique France.
**Fig. 2.** Phylogenetic tree to the ITS-5.8S region indicating the positioning of the *Agaricus* sp. (bold) within the *Agaricus subrufescens*. Bootstrap values on nodes correspond to Maximum likelihood and Neighbour joining respectively. Midpoint rooting. Bar = number of replacements by position. BR = Brazil; CHN = China; KP = North Korea; COL = Colombia; EC = Ecuador; FR = France; GER = Germany; IND = India; THA = Thailand and UK = United Kingdom.

**Fig. 2.** Árbol filogenético de la región ITS-5.8S que indica el posicionamiento de *Agaricus* sp. (negrita) dentro del clado *Agaricus subrufescens*. Valores de Bootstrap sobre los nodos corresponden a Máxima verosimilitud y Vecino más cercano respectivamente. En-raizamiento en punto medio. Barra = número de sustituciones por posición. BR = Brasil; CHN = China; KP = Corea del Norte; COL = Colombia; EC = Ecuador; FR = Francia; GER = Alemania; IND = India; THA = Tailandia y UK = Reino Unido.

**Taxonomy**

*Agaricus subrufescens* Peck, Ann.
Figs. 3 A-K

**Basidiome** agaricoid, solitary with a slender, elongated stipe and a convex whitish-yellowish pileus with slightly reddish fine-grained scales. Growing on soil with abundant humus-rich mantle and leaf litter. **Pileus** 8–9 cm in diameter; convex with a tendency to flatten according to development, slightly scaly and floccose, yellowish-white (#d8d6cf) to reddish in colour,
more concentrated in the central part of the pileus (#b5948e). Lamellae free, strongly narrowed, pinkish (#bb9b90) when young and brown (#79615b) when come old. Stipe 10 cm long and 1 cm wide; ribbed sub-cylindrical; smooth and white, with bulbous base 1.30 cm wide generated from abundant mycelium on the substrate. Upper ring attached to the hymenium, with hanging white membranous hymenial veil, which is smooth upper side and squamulose lower side. Sporeprint dark brown (#877459).

Macrochemical reactions.— Schäffer reaction not tested, KOH 10% reaction positive (yellowish).

Basidiospores.— 5.5–6.5 × 3.5–4.5 μm, Q= 1.5, elliptical to suboblong with thick-walled; light brown (#a49074) to dark brown (#877459). Basidia 13.5–16 × 5.6–7 μm; clavate; tetrasporic; thin-walled; hyaline. Cheilocystidia 7–12 × 6–10 μm; subglobose; simple or catenulate; hyaline; thin-walled; frequent to abundant. Pleurocystidia not observed. Pileipellis cylindrical hyphae 3–12 μm diam.

Geographical distribution.— Usually reported to (sub) tropical broadleaf cloud forests Medel-Ortiz et al. (2022) in América, but also reported in Europa and other continents (Table 1). Here is the first record for Ecuador and El Oro-Loja province.

Specimen examined.— ECUADOR. El Oro-Loja; Puyango-Alamor; from outer limit Puyango Petrified Forest; Saprotrophic on humus-rich mantle and leaf litter, 29-May-2021, Cruz, D., BP001, HUTPL(F)2047.

Remarks.— The studied specimen Agaricus subrufescens BP001 HUTPL(F)2047 macromorphologically agree with the description of the species reported from Mexico (Velázquez-Narváez et al., 2018) and Dominican Republic (Medel-Ortiz et al., 2022). Microscopically, the studied specimen presents, and elliptical to suboblong basidiospores: 5.5–6.5 × 3.5–4.5 μm, slightly different from Agaricus subrufescens described by (Peck, 1893; in the protologue) which registers a pileus of 5–8 cm diameter, a stipe 5–15 cm long and 0.8 cm wide in Peck and brown elliptic basidiospores of approximately 6–7 μm × 4–5 μm. Our specimen is more similar to the specimen described from Mexico by Velázquez-Narváez et al. (2018) specially for their basidiospore 5.6–6.9 (7.5) × 3.5–4.6 (4.9) μm, and also similar to the basidiospores measurements record for A. fiardii 4.7–6 × 3.2–4.5 μm (Medel-Ortiz et al., 2022).

DISCUSSION

The morpho-molecular analysis carried out in this study confirms the assignment of our specimen as Agaricus subrufescens, this being the first time that its presence has been reported in Ecuador, which expands the knowledge about its distribution. The specimen (Fig. 3), was found on the outer perimeter of the Puyango Petrified Forest (PPF) which is into a natural tropical dry forest, characterized by remnant endemic vegetation and an ecosystem of petrified trunks (Jumbo-Eras et al., 2021).

Molecular phylogenetic inference

Phylogenetically our sequence (ON799184) of Agaricus subrufescens is cluster together several sequences: A. blazei from North Korea (KP) AF161013
and Brazil (BR) AY484697; A. subrufescens from Brazil (BR) KJ541796; A. rufotegulis from United Kingdom (UK) AY818649; A. fiardii from France (FR) JF797201; and Agaricus sp. JF514529 of Thailand (THA) in one clade with 99% support in the Neighbor-Joining (NJ) and Maximum Likelihood (ML) analyses (Fig. 2). These taxonomic relationships have been widely discussed by Kerrigan (2005) and Medel-Ortiz et al. (2022).

The clade formed with sequences mostly assigned to A. subrufescens (Fig. 2) and other Agaricus species, was considered as species complex clade “A. subrufescens” by Zhao et al. (2011). This clade (Fig. 2) includes a sequence obtained from a basidiome identified as Agaricus fiardii voucher F2285 LIP Martinique France, (GenBank accession number JF514529) (Zhao et al., 2011). This species A. fiardii was originally described from Martinique France by Pegler (1983) and it is morphologically like A. subrufescens. Additionally when compare phylogenetically the ITS2 sequences, one clade is generated (data do not show) clustering sequences from A. fiardii type material [K(W)234316], and sequences to A. brasiensis; A. blazei; and others (Table 1) which are considered synonyms in many studies (Wasser et al., 2002; Kerrigan, 2005; Thongklang et al., 2016; Zied et al., 2021; Medel-Ortiz et al., 2022).

Surprisingly one sequence named as Agaricus campestris voucher NVE470, (GenBank accession number KF937296) isolated from basidiome is clustered into this clade. This basidiome was found in Boyacá-Arcabuco, Colombia and referenced according to GenBank information to the work of Vasco-Palacios et al. (2014). However, in Vasco-Palacios et al. (2014) no morphological information or sequence analysis is presented for this specimen. So that, in the present study this sequence and species are considered as a probable misassignment.

Agaricus subrufescens, has been widely discussed at taxonomic level (Wasser et al., 2002; Kerrigan, 2005; Thongklang et al., 2016; Zied et al., 2021; Medel-Ortiz et al., 2022), and is considered as an important edible fungus with medicinal properties (Hetland et al., 2011). Their efficiency in the immune system against allergies, tumours and other health problems is attributed to the generation of secondary metabolites mainly β-glucan polysaccharides (Hetland et al., 2011). Therefore, this fungus is commercially cultivated in different American, European countries and other continents (Wisitrassameewong et al., 2012; Thongklang et al., 2016; Velázquez-Nárñáez et al., 2018; Zied et al., 2021).

This finding strengthens Ecuador as Megadiverse, with fungi in tropical areas with edible potential, unlike other fungi that are mainly known for high Andean areas used for indigenous communities (Andrade et al., 2012; Gamboa-Trujillo et al., 2019). Therefore, further exploration and knowledge of fungal diversity is needed, and it should also be included in conservation policies, as is currently the case for endemic or endangered species of flora and fauna (Albán, 2010). These new non-timber forest resources have potential to be exploit biotechnologically.
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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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