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Technical note

# Psychedelic fungus (*Psilocybe* sp.) authentication in a case of illegal drug traffic: sporological, molecular analysis and identification of the psychoactive substance

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ABSTRACT

In nature, there are > 200 species of fungi with hallucinogenic properties. These fungi are classified as Psilocybe, Gymnopilus, and Panaeolus which contain active principles with hallucinogenic properties such as ibotenic acid, psilocybin, psilocin, or baeocystin. In Chile, fungi seizures are mainly of mature specimens or spores. However, clandestine laboratories have been found that process fungus samples at the mycelium stage. In this transient stage of growth (mycelium), traditional taxonomic identification is not feasible, making it necessary to develop a new method of study.

Currently, DNA analysis is the only reliable method that can be used as an identification tool for the purposes of supporting evidence, due to the high variability of DNA between species. One way to identify the species of a distinctive DNA fragment is to study PCR products analyzed by real time PCR and sequencing. One of the most popular sequencing methods of forensic interest at the generic and intra-generic levels in plants is internal transcribed spacer (ITS). With real time PCR it is possible to distinguish PCR products by differential analysis of their melting temperature (Tm) curves.

This paper describes morphological, chemical, and genetic analysis of mycelia of psychedelic fungi collected from a clandestine laboratory. The fungus species were identified using scanning electron microscopy (SEM), mass spectrometry, HRM analysis, and ITS sequencing. The sporological studies showed a generally smooth surface and oval shape, with maximum length 10.1 μm and width 6.4 μm. The alkaloid Psilocyn was identified by mass spectrometry, while HRM analysis and ITS sequencing identified the species as *Psilocybe cubensis*. A genetic match was confirmed between the HRM curves obtained from the mycelia (evidence) and biological tissue extracted from the fruiting bodies. Mycelia recovered from the evidence and fruiting bodies (control) were genetically indistinguishable.

## 1. Introduction

There are > 200 fungal species with narcotic properties classified in the genera Psilocybe, Gymnopilus, Panaeolus, Agrocybe, Conocybe, Copelandia, Galerina, Gerronema, Hypholoma, Inocybe, Mycena, and Plutes that may contain the active principles ibotenic acid, psilocybin, psilocin, and baeocystin [1,2]. In recent years, the consumption of natural biological products with hallucinogenic effects has increased. These products include easy-access narcotics such as fungi, which are used for recreational and mystic purposes [3]. Poisoning and dangerous psychedelic effects caused by psilocin and its derivatives still constitute a major medical and social problem, mainly among young individuals. Therefore, quick and reliable identification of these substances is of crucial forensic interest [3].

In *P cubensis*, the psilocin content range is 0.17–0.78% in the cap and 0.09–0.30% in the stem while the psilocybin content range is

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0.44–1.35% in the cap and 0.05–1.27% in the stem [4]. Moreover, other authors also reported a higher content of psilocybin when compared to psilocin in *P. cubensis* [4,5]. Psilocybin, which has entheogenic properties, is found mainly in Psilocybe fungi [5]. One classic method to produce fungi is through fungal culture [6]. The development of fungi in culture include the following stages: a) teleomorph (fruiting body), b) anamorph (asexual stage), and c) holomorph [6]. Spores may be produced either directly by asexual methods or indirectly by sexual reproduction. Perhaps the simplest method of reproduction of fungi is by fragmentation of the thallus, the body of a fungus. Several fungal cultures have been detected in the transient mycelium stage. Taxonomic identification during the transient mycelium stage is very challenging, requiring the use of additional DNA-based techniques for this purpose. Many countries, however, have some level of regulation or ban of psilocybin mushrooms (for example, the US Psychotropic Substances Act, the UK Misuse of Drugs Act 1971, and the Canadian Controlled Drugs and Substances Act) [7,8]. The ban of psilocybin mushrooms has come under criticism, from the public and from researchers, who see therapeutic potential regarding drug addictions and other mental instabilities, such as post-traumatic stress disorder (PTSD). Among regulated drugs, psilocybin mushrooms have relatively few medical risks. Legal regulations of psilocybin mushrooms by country is variable, for example it is illegal in United Kingdom, United State, Japan, Mexico, Australia, France, Germany, and Denmark. It is legal in Brazil, Jamaica, and Canada (possession and sale) [7,8].

Thus, fungal identification using PCR-based methods would provide a forensic tool for law enforcement agencies [9]. Different molecular techniques have been applied to analyze fungal DNA including *Psilocybe* species. A previous study by Schoch et al. [10] recommended the nuclear ribosomal RNA internal transcribed spacer (ITS) region as a target for universal DNA barcode for fungi. ITS is recognized as an ideal fungal barcode because it is the most sequenced region of fungi and is routinely used for systematics, phylogenetics, and identification [10]. ITS barcoding exhibited the highest probability of correct identification (PCI) for a wide number of fungal lineages analyzed and the most clearly defined barcode gap. Badotti et al. [11], analyzed one of the most suitable genomic markers (complete ITS, ITS1 or ITS2) for identification of fungal species belonging to Basidiomycota. This study reported a probable correct identification (PCI) estimated values of 100% for complete ITS genomic regions (IST + ITS1 + 5.8S + ITS2), sub-region ITS1 and, for sub-region ITS2, for the division Basidiomycota, class Agaricomycetes, order Agaricales, family Agaricaceae, genera Psilocybe. DNA barcoding data can be generated with real-time PCR combined with high resolution melting (HRM) analysis to distinguish specific conserved DNA regions of closely related species [12;13]. High-resolution melting (HRM) analysis is a method of choice for rapid analysis of sequence variation within PCR amplicons [14]. Genetic variants in the genomic base composition display differences in their melting temperatures. Melting temperature differences can be detected by monitoring the fluorescence changes as the temperature is increased during PCR. Species are then differentiated by their characteristic melting curves, visualized by the loss of fluorescence as the DNA duplex melts [15]. High-resolution melting (HRM) analysis allows genotyping of fungus species by differentiation of DNA sequence variants such as single nucleotide polymorphisms (SNPs) and small insertions and deletions (indels), based on the location of a differential peak and shape of the melting transition curves (Tm) [12–14]. HRM has emerged as a useful molecular tool in several genetic areas due to its specificity, speed, and affordable cost [11].

This paper described a case of a drug seizure from a fungi clandestine laboratory. Glass flasks were confiscated from the laboratory, containing rice grains covered with an unidentified white fibrous material suspected to be the hallucinogenic fungus *Psilocybe* sp. The objective of this work was to identify psychedelic fungi collected from a clandestine laboratory by sporological study, biochemical analysis, and molecular study of mycelia and fruiting bodies.

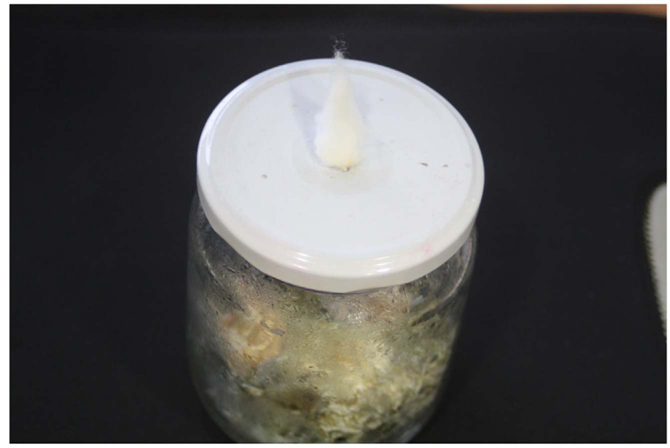

**Fig. 1.** Transitional mycelium obtained from bottles (seizure evidence).

## 2. Materials and methods

### 2.1. Case of drug trafficking

Fungi samples were seized from a clandestine laboratory in La Araucanía Region in southern Chile (Fig. 1). The sample of *Psilocybe* fungi were taken from transitional mycelia obtained from bottles used as growth chambers. The growth of the fruiting body was induced for morphotaxonomic confirmation. Mycelia and biological tissue extracted from the fruiting bodies of *Psilocybe* sp. were used for ITS molecular analysis. The *Lepthosphaería maculans* (Desmaz.) Ces. & De Not. DNA (M3 isolate) was used as control for ITS molecular analysis.

### 2.2. Sporological studies

Spores were collected by leaving the umbrella in contact with a sheet of paper for 12 h, during which time the spores were transferred to the paper. A sample was fixed with a piece of tape and analyzed directly using SEM microscope FEI™, 3200× magnification.

### 2.3. Identification of psychoactive substance

The hallucinogenic active principle was identified by gas chromatography coupled with mass spectrometry (GC–MS). The active principle was extracted with methanol from basic media using 300 mg of tissue from the fungus fruiting body, sonicated for 20 min at room temperature, and centrifuged at 2200 rpm for 3 min. The supernatant was dried, re-suspended in 300 μL of ethanol and injected in a GC–MS AGILENT™, using Acq Method Rastreomcd. M.

### 2.4. DNA extraction and HRM-RTPCR

Genomic DNA was isolated by DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. Samples were quantified with a Qubit 2.0 fluorometer (Invitrogen). PCR amplification, DNA melting, and fluorescence level acquisition for the PCR amplifications were performed in a total volume of 15 μL on an Illumina real-time PCR Thermocycler (ILLUMINA-ECO™Real-Time PCR System). SYBR Green I was used to monitor the accumulation of the amplified product during PCR and subsequent product melting in the Illumina Thermocycler (Eco™ Software v 4.1.2.0). The reaction mixture contained 20 ng genomic DNA, 300 nM primers (Table 1) [19], and Fast PCR Master MIX SYBR green I 2× (Kapa Biosystems, USA). The ITS-PCR protocol was performed using an initial denaturing step of 94 °C for 2 min followed by 35 cycles of 94 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension step of 72 °C for 2 min. The fluorescence data were acquired at the end of each extension step during PCR cycles. Before

**Table 1**
Primers used for ITS sequencing and HRM analysis of *Psilocybe* sp.

| Marker | Primer | Sequence (5′→ > 3′) | Reference |
|---|---|---|---|
| ITS region | ITS1 | 5′ TCCGTAGGTGAACCTGCGG 3′ | [19] |
| | ITS4 | 5′ TCCTCCGCTTATTGATATGC 3′ | [19] |

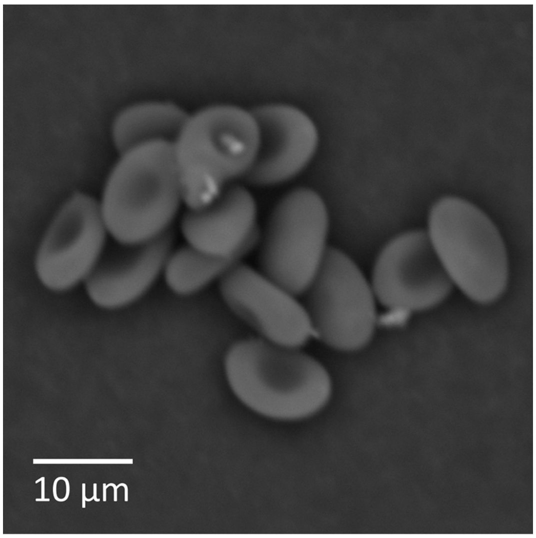


**Fig. 2.** Spores of *Psilocybe* sp. under SE microscope FEI™, with 3200× magnification.

HRM, the products were denatured at 95 °C for 5 s, and then annealed at 50 °C for 30 s to form random DNA duplexes. HRM was performed as follows: pre-melt at the first appropriate temperature for 90 s, and melt at a ramp of 10 °C in an appropriate temperature range with 0.1 °C increments every 2 s. Finally, the 2-resolution melt curve (HRM) of ITS markers was obtained using 95 °C for 15 s, 50 °C for 15 s and 95 °C for 15 s. The melting pattern of corresponding sequences of ITS amplicons from samples were analyzed using the computer program Eco™ Software v4.1.2.0.

### 2.5. Sequence analysis

PCR products were directly sequenced in two directions for each product using a BigDye Terminator V3.1 Cycle Kit in an automated Genetic Analyzer 3500 xl (Thermo Scientific), with software Data Collection v3. Sequences were aligned and proof-read using the software MEGA 5 and submitted to GenBank (Accession KU640170.1).

## 3. Results

### 3.1. Sporological studies

Scanning electron microscopy images of the spores showed a generally smooth surface and an oval shape, maximum length 10.1 µm and width 6.4 µm (Fig. 2). These values coincide with those reported for the fungus *Psilocybe cubensis* [5].

### 3.2. Identification of the psychoactive substance

The extracts of the psychoactive substances from the fungus were identified by their mass spectrum. The mass spectrum and their retention times were compared with data available in the library NIST 08.L. GC–MS results of the analysis carried out on fruiting body tissue correspond to psilocin, an alkaloid derived from psilocybin which has entheogenic properties and is found mainly in Psilocybe fungi (Fig. 3) [4;5].

### 3.3. Molecular identification

As expected, PCR of mycelium and fruiting bodies (*Psilocybe* sp.) were successful. Both structures provide abundant DNA for PCR analysis, which is important when the available evidence consists of structures of transitory developmental stages of the fungi. The DNA melting profile was analyzed to investigate whether polymorphism in the ITS region of different states of the fungus is detectable in derivative and normalized melting curves (Fig. 4a and b). Only one species was distinguished visually in normalized HRM curves of the ITS region, with HRM curves which were highly characteristic for amplicons in the range 82.0 °C to 85.0 °C. They are dependent on the interplay between GC content, length of amplified product and sequence. All profiles of

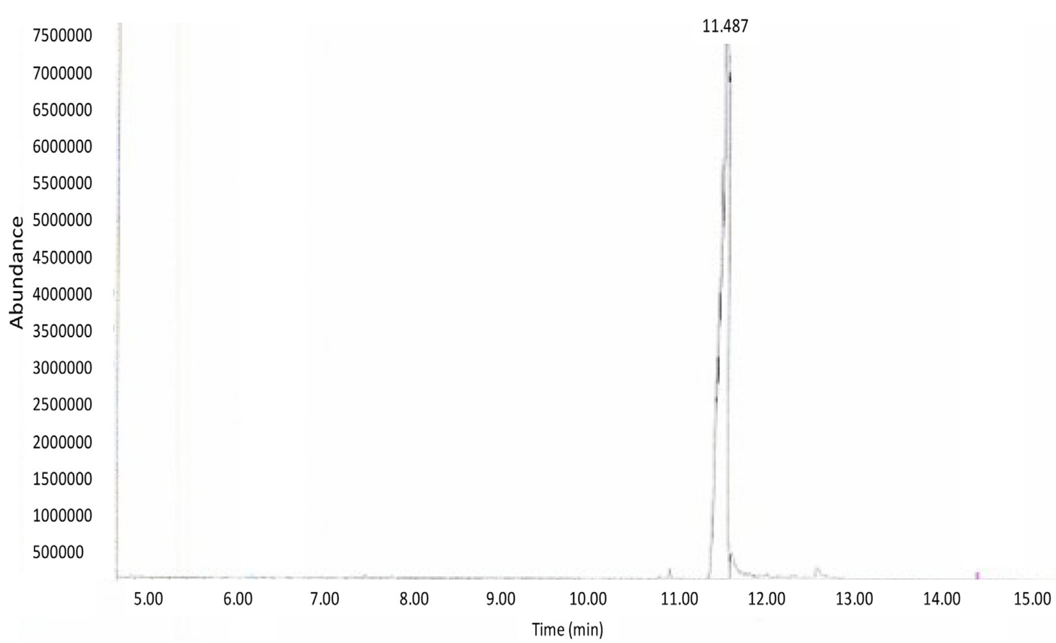


**Fig. 3.** GC–MS results of the analysis carried on fruiting body.

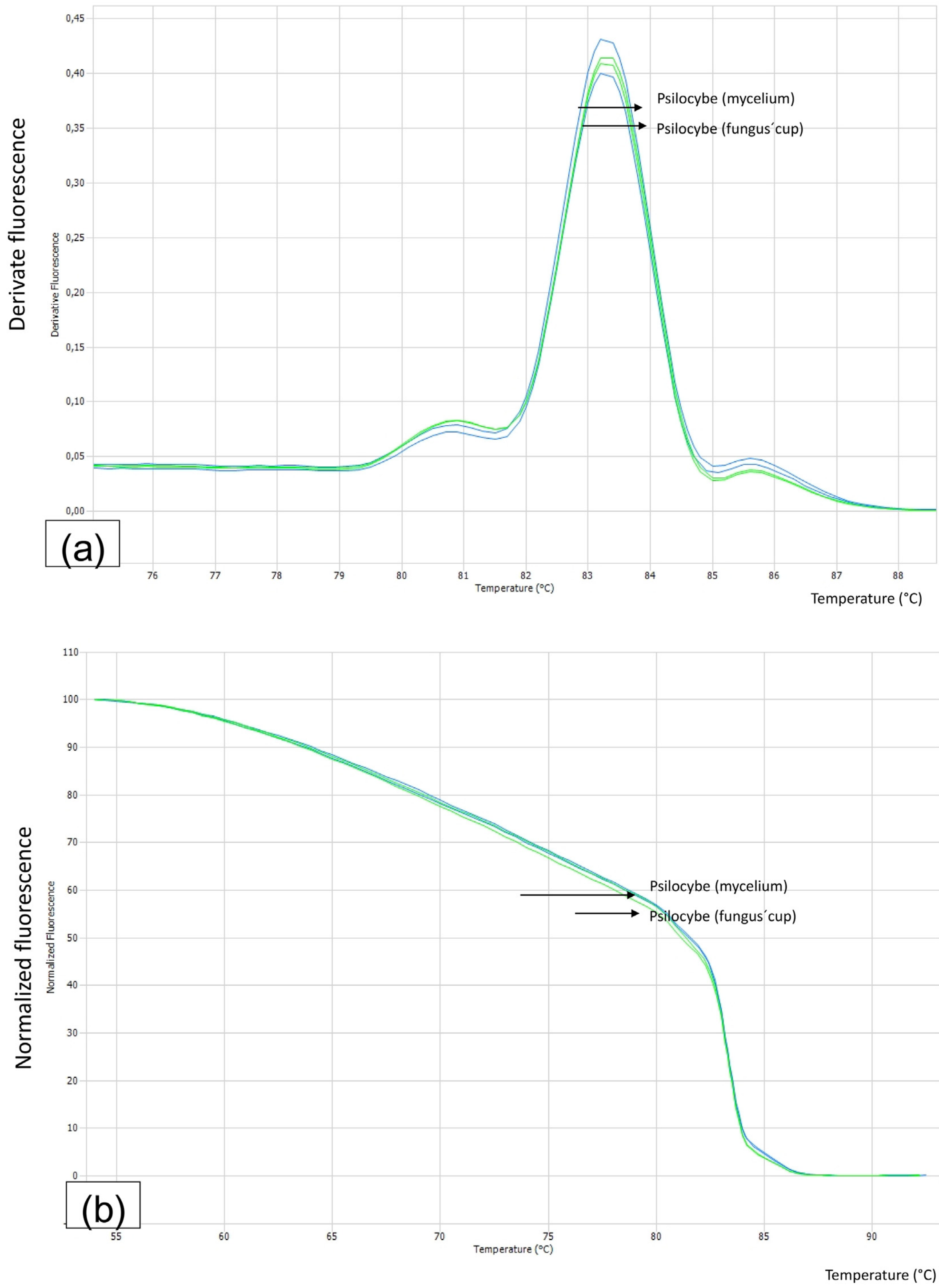


**Fig. 4.** (a) Normalized melt curve; (b) Derivative melt with high-resolution melting (HRM) with ITS approaches for *Psilocybe* sp.

*Psilocybe* sp. (mycelium and fruiting bodies) produced one peak. The HRM melting curves were similar, with the same Tm values of 83.30 °C for mycelium and fruit bodies. The samples were genetically indistinguishable and therefore corresponded to the same genotype. All profiles of *Psilocybe* sp. (mycelium and fruiting bodies) produced one peak. The melting curves are indent in shape. We found that the ITS marker is a potential region for distinguishing different structures and states of development. The graph of the normalized data (Fig. 4a) shows more consistent melting profiles in the *Psilocybe* samples evaluated. We used *Lepthosphaería maculans* DNA used as control, amplified fragments from 524 to 550 bp. The graph of the normalization data (Fig. 5a) shows more consistent melting profiles in the Psilocybe samples evaluated. This allowed better grouping of the curves of different samples in the graph, and improved differentiation between *Psilocybe* sp. and *L. maculans*. The melting curves are different in shape, and the Tm of Pilocybe is approximately 2.05 °C lower than *L. maculans* (ΔTm = 2.05 °C). Furthermore, the melting curve of *L. maculans* shows a gradual transition over a wide temperature range and Tm of 85.35 °C (Fig. 5b).

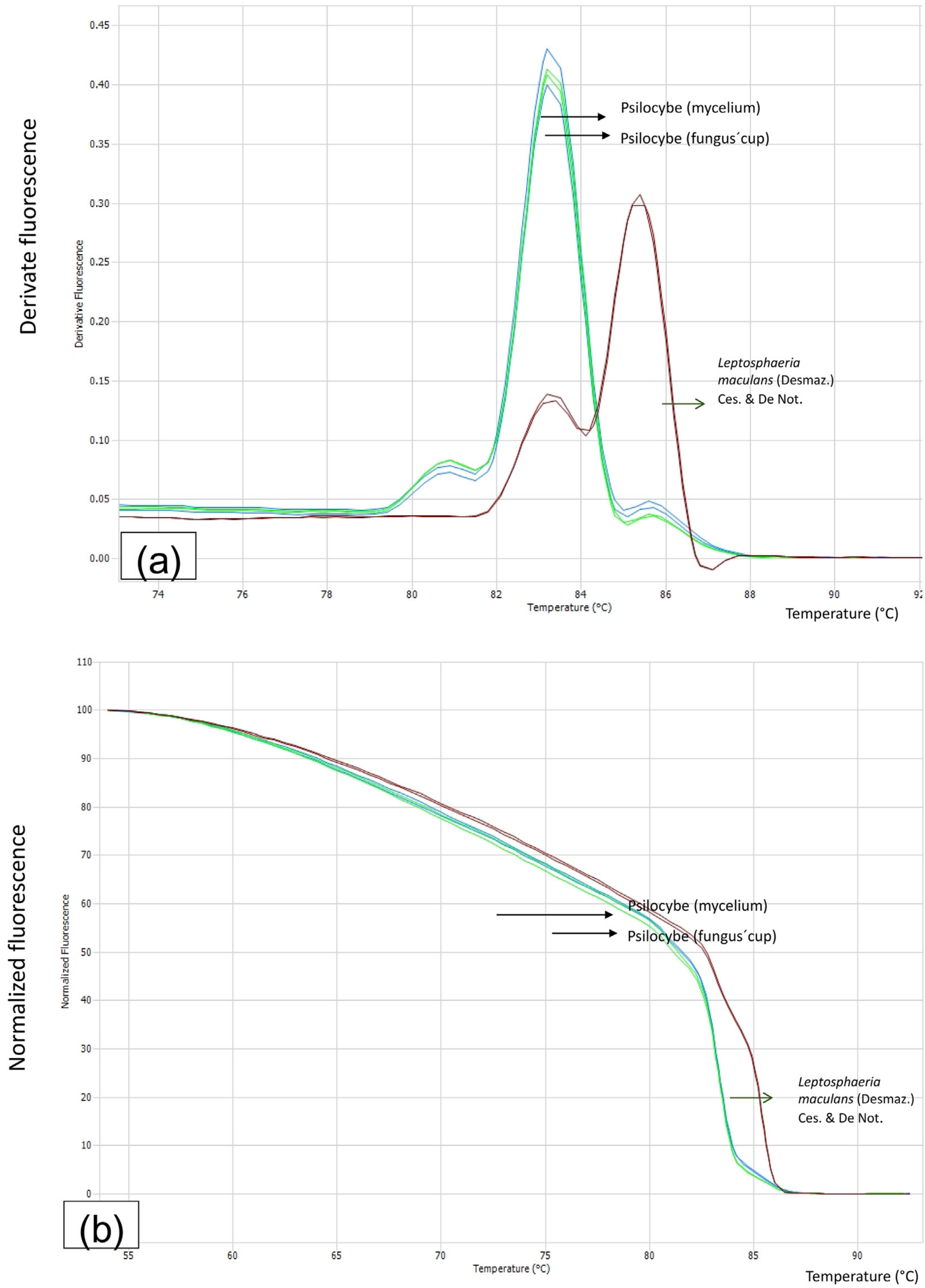


**Fig. 5.** (a) Normalized melt curve; (b) Derivative melt with high-resolution melting (HRM) with ITS approaches for *Psilocybe* sp. and *L. maculans*.

### 3.4. Sequence analysis

Results were also confirmed by sequence analysis of the ITS region, which revealed that only one species was present (*P. cubensis*). After analysis of the sequences from ITS regions (ITS1) with different families and species registered at GenBank, a sequence of 715 bp was obtained. BLAST analysis of the fruiting body of the hallucinogenic fungus showed 99% coincidence with the species *Psilocybe cubensis* (accession KU640170.1), order Basidiomycota, Agaricomycotina [20]. Alignment of isolations of *L. maculans* (524 bp) obtained from raps crops showed a 89% agreement with accession GU205260.1, belonging to the Ascomycota *Leptosphaeria maculans* "brassicae" group. On the other hand, the degree of similarity of the amplified sequences of the ITS region with other nearby fungal species were: *Galerina clavate,* 98%, *Psilocybe chuxiongenesis,* 96%, *Psilocybe subcubensis,* 98%, *Psilocybeovoideocystidiata,* 91%, *Psilocybe subaeruginascens,* 90% for ITS1, and *Psilocybe clavate,* 99%, *Psilocybe chuxiongenesis,* 97%, *Psilocybe subcubensis,* 99%, *Psilocybe ovoideocystidiata,* 92%, *Psilocybe subaeruginosa,* 91% for ITS4.

## 4. Discussion

We described a case in which a fungus species was identified using spore morphology, mass spectrometry, HRM analysis and sequence with ITS approach. Mycelia and fruiting bodies of psychedelic fungi collected from a clandestine laboratory were examined. Scanning electron microscopy images of the spores coincides with those reported by Tsujikawa et al. (2003) [5] to describe *Psilocybe cubensis*, with dark brown spores of dimensions 10.2–16.5 μm × 5.9–10.0 μm. GC–MS results of the analysis carried out on fruiting body tissue identified psilocin, as in the reports of other authors [4]. Tsujikawa et al. [5] determined the content of this alkaloid by HPLC after extraction with methanol and reported that the psilocin/psilocybin content in *Psilocybe cubensis* is more often contained in the cap than the stem, with larger amounts of psilocybin than psilocin. Molecular authentication showed that both structures of the fungus (mycelium and fruiting bodies) are good sources of DNA, providing full and amplifiable genetic material that can be used efficiently for authentication analysis by HRM and sequencing [9]. HRM involves accurate, precise monitoring of fluorescence changes caused by the release of an intercalating DNA dye from double stranded DNA during its denaturation caused by increased temperatures. HRM analysis has been used for the identification of fungi by different authors [16;17;18;21]. Confirmation of the results by sequencing analysis of the ITS region showed that *Psilocybe cubensis* was the only species present. Comparative sequence analysis confirmed the power of HRM analysis to distinguish the fungi and authenticate different structures such as mycelia and fruiting bodies. Detection of the specific DNA sequence for *Psilocybe semilanceata* is commonly employed in forensic practice [5].

The similarity observed between the amplified sequences and other nearby fungal species fluctuated between 91 and 99%. This could limit the application of the DNA Barcoding strategy for the authentication of hallucinogenic fungi, however, the present investigation considered the integration of different analytical techniques, which were independent in the fungus identification. For a case of mixing Psilocybe with another type of fungus (non-psilocybe), a mixture of spores of different morphology would surely be observed under a microscope. For example *P. subcubensis* was classified as a different species from *P.cubensis* by Guzman 1978 [22]. These two species are macroscopically and even microscopically very similar, with a slight difference in spore sizes. Regarding the GC–MS, if there was another fungus in the mixture, a mixture of different psychoactive compounds would be showed in the chromatogram. In the HRM, if the fungi of the mixture have different sequences, numerous temperatures of melting and shapes curves would be observed. In the case of sequencing, mixtures cannot be sequenced unless done through next generation sequencing.

HRM analysis using ITS allows early forensic authentication of the fungus in the mycelium stage. The ITS regions of basidiomycete fungi present specific characteristics [11] with applications for DNA barcode marking. Additionally, the reliability, high speed, and lower cost of DNA analysis mean that genetic methods are more often used to identify fungal species. These methods are random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and high-resolution melting (HRM). Moreover, it has been suggested that analysis of the ITS1 and nLSU regions is a valid method for application in the molecular taxonomy of fungi for forensic purposes [3]. The molecular results coincide with the microscopic analysis of spores and with the identification of the psychoactive substance found in the fruiting bodies tissue, psilocin. These results are similar to those of Anastaso et al. [1], which showed that the useful life of the standard is increased significantly, producing stable solutions of psilocin and psilocibyn up to seven days. Comparison of the biochemistry technique used in this study with that performed by Dydak et al. [3] allowed identification of psilocybin and psilocin in fungi and biological material [3]: zone capillary electrophoresis, high performance liquid chromatography, gas chromatography and liquid chromatography coupled with mass spectrometry. These methods are successfully used to identify psychoactive substances in fungi as well as in blood and urine samples [3]. In conclusion, the seized evidence corresponds to *Psilocybe cubensis*, a mushroom with hallucinogenic properties in its first stage of growth (mycelium). The fungus species was identified using microscopic examination and confirmed with HRM analysis and sequencing. This latter approach is a fast, low cost, easy-to-use forensic tool, which allows fungus fragments to be identified in different development stages. Finally, molecular biology techniques can help to identify illegal crops of psychedelic fungus and can be used in court in drug trafficking cases.

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## Conflict of interest

The authors declare no conflict of interest.

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