Botanical DNA evidence in a case of illegal drug traffic: Use of High Resolution Melting (HRM) with Internal Transcribed Spacer (ITS) approach in the identification of psychedelic fungus (Psilocybe sp.).

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ABSTRACT

Forensic botany is an emerging discipline that has evolved rapidly during the last few years. Botanical evidence is usually found at crime scenes and sometimes it is the only available element for criminal investigations.
Currently, as supporting evidence, DNA analysis is practically the only element that can be used as a reliable identification tool, due to the high variability of DNA nature across all species.
One way to identify a distinctive DNA fragment for a specie is the study of PCR products analyzed via real time PCR. With this technique is possible to distinguishing PCR products using their melting temperature (Tm) curves differential analysis. One of the most popular sequences of forensic interest at the generic and intra-generic levels in plants is the internal transcribed spacer (ITS).
In Chile, fungi seizures are mainly composed of mature specimens or spores. However, it was found that clandestine laboratories processed fungus samples at the stage of mycelium. In this transient stage of growth (mycelium), traditional taxonomic, microscopy and chemical identification are not feasible, making it necessary to develop a new method of study.

INTRODUCTION

In recent years, the recreational use of the hallucinogenic mushroom, so-called “magic mushroom”, has become an increasing social problem in several countries. In nature, there are more than 200 species of fungi with hallucinogenic properties (Psilocybe, Gymnopilus and Paneolus) and they contain active principles such as: ibotenic acid, psilocybin, psilocin or baeocystin. At the present time they are not only naturally occurring but also offered as a kits for cultivation.
The cultivation or possession of psicodelyc mushrooms have been controlled by the Drugs Control Law in Chile since 2014.The case described in this paper refers to the genetic analysis of mycelia of psychedelic fungi that was found by the police and collected from a clandestine laboratory in Temuco city in Chile.

The identity of fungus species was achieved using High Resolution Melting (HRM) analysis and ITS approach. Genetic variants with differences in the base composition present differences in their melting temperatures. These are detected by monitoring the fluorescence as the temperature increased, and the species are differentiated by their characteristic melting curves, visualized by the loss of fluorescence as the DNA duplex melts (Nicklas et al., 2012). The suspect was arrested and convicted for the crime.

MATERIALS

SAMPLING

The first samples were taken from transitional mycelium obtained from glass bottles used as growth chamber. Then, 10 g mycellium evidence was mixed with 200 g wet vermiculite in plastiic container of 500 ml capacity. The sides container were pierced with holes of 1 cm diameter, and covered with cotton. Finally , after 15 days little fruit body mushroom begin grow up in the pack.

METHODS

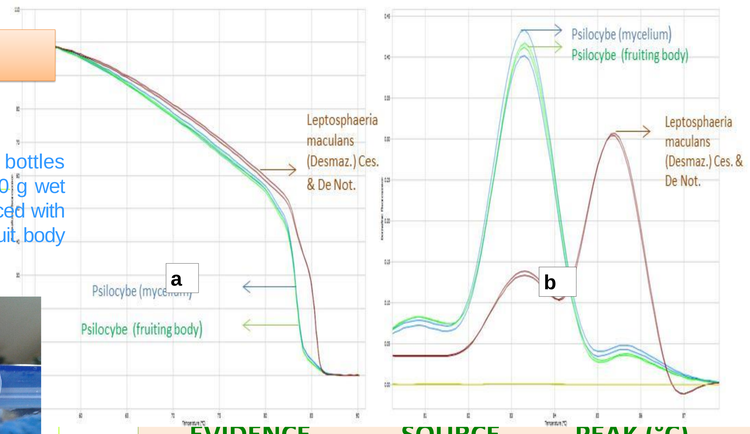
PROCEDURE

Genetic analysis: Fungus DNA was isolated from mycelium and biological tissue of the mushrooms´ cap according by White *et, al*., (1990) following the manufacturer's instructions and quantified by fluorimetry. HRM analysis of dna using ITS 1-4 primers was performed in a Real-time PCR Thermocycler (ILLUMINA-ECO™ Real Time PCR System). DNA from Lepthosphaería maculan fungus was used as control for ITS molecular report.

Morphologic specie identification complementary: SEM microscopy examination was carried out to analize spores and identified the specie. Spores collection was carried out putting down the umbrella resting on a paper sheet by 12 hours. When the umbrella was taken up spores were found finely spread over the paper support. A sample was fixed with a piece of tape and was analyzed directly using a SEM microscope FEI™, 3200x magnification.

Figure 2. ILLUMINA-ECO™ Real-Time PCR System.

RESULTS & DISCUSSION



HRM and ITS *aculans*.

EVIDENCE SAMPLE	SOURCE OF DNA	PEAK (°C) ± SD	PEAK (°F) ± SD
Psilocybe sp.	Mycelia	83.30 ± 0.00	181.94 ± 0,00
Psilocybe sp.	Fungus cap	83.30 ± 0.00	181,94 ± 0.45
L. maculans	Isolation M3	85.35 ± 0.07	185,63 ± 0.45

Table 1. Variability for the melting peak of the amplicons from *Psilocybe sp.* and *L. maculans* sample with ITS by high-resolution melt (HRM).

RESULTS & DISCUSSION

Morphologic spore analysis. Scanning electron microscopy images of the spores showed a general smooth surface and oval shape with a center hole, maximum length of 10.1 mm and width of 6.4 mm, being the length of the fissure of 3.9 mm and its width of 2.7 mm, which are characteristics coincident with the reported for fungi of *Psilocybe* sp. (Kenji Tsujikawa et al, 2003).

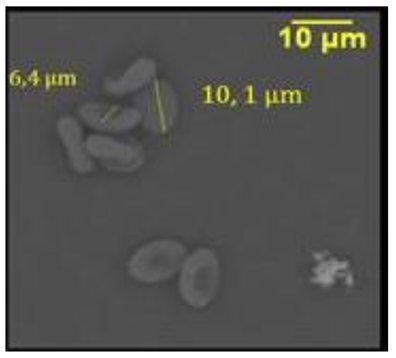


Figure 4. Psilocybe sp. spores.

Genetic analysis: PCR process for fungus evidences and control was successful, and it is possible because both mycelium and fruit body supplied plenty genetic material, which is important when the evidences are limited.
Analysis of the normalized HRM curves with ITS region revealed that two species could be distinguished visually, based at their shapes and temperature peaks (Tm) characteristic. *Psilocybe sp* mushrooms is 3.96 ˚ F lower than *L. maculans* fungus control. A genetic match was confirmed between the HRM curves obtained from the mycelium and biological tissue extracted from the fungus’ cap, generating a unique peak at 181,94 ˚ F (Tm).

CONCLUSION

The seizured evidence corresponds to *Psilocybe sp*. mushroom with hallucinogenic properties in its first stage of growth (mycellium). The identity of fungus species was achieved using microscopic examination (SEM) but in the present investigation was established that High Resolution Melting analysis and ITS approach is an economic, easy to use and fast forensic tool that allows the detection of fungi fragments in different states of development unlike other techniques which require mature fungi or spores.
The genetic study of botanical evidence collected at clandestine laboratory contributed to the reconstruction of the illegal event and provided elements to be used in court.

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