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Forensic analysis of hallucinogenic fungi: a DNA-based approach☆

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Abstract

Hallucinogenic fungi synthesize two controlled substances, psilocin and psilocybin. Possession of the fungal species that contain these compounds is a criminal offence in North America. Some related species that are morphologically similar, do not contain the controlled substances. Therefore, unambiguous identification of fungi to the species level is critical in determining if a mushroom is illegal. We investigate a phylogenetic approach for the identification of species that contain the psychoactive compounds. We analyzed 35 North American specimens representing seven different genera of hallucinogenic and non-hallucinogenic mushrooms. We amplified and sequenced the internal transcribed spacer region of the rDNA (ITS-1) and a 5′ portion of the nuclear large ribosomal subunit of rRNA (nLSU rRNA or 28S). ITS-1 locus sequence data was highly variable and produced a phylogenetic resolution that was not consistent with morphological identifications. In contrast, the nLSU rRNA data clustered isolates from the same species and separated hallucinogen containing and non-hallucinogen containing isolates into distinct clades. With this information, we propose an approach that combines the specificity of PCR detection and the resolving power of phylogenetic analysis to efficiently and unambiguously identify hallucinogenic fungal specimens for legal purposes.

Keywords: Hallucinogenic fungi; ITS; nLSU rRNA; DNA identification

1. Introduction

Forensic DNA analysis has revolutionized the way in which biological samples may be used as irrefutable evidence in legal proceedings. The basis of these identifications is variation in nucleotide sequence. Technological advances that speed analyses of sequence variation make DNA profiling a practical choice for the unambiguous identification of individuals and species. Human nuclear DNA has proven successful in determining paternity as well as identifying individuals from semen, hair or teeth collected as evidence. DNA-based approaches have also been proposed for the identification of marijuana, blowfly specimens and illicit fungal genera [1–4]. We have investigated a phylogenetic approach that allows the identification of fungal species containing controlled substances.

Hallucinogenic fungi taxonomically fall into several genera, the most well known being the genus *Psilocybe*. Certain species of these genera synthesize psilocin and psilocybin, which are the illegal compounds that produce mind-altering effects. In Canada, these compounds are classified as restricted drugs and are listed in Schedule III of Canada's Food and Drugs Act [5]. The penalty for possession ranges from CAN$ 1000 and/or 5 months imprisonment for a first offence, to CAN$ 5000 and/or 3 years imprisonment for more serious offences. In comparison, within the US, any act involving the substances psilocybin and psilocin is considered to be a crime under both federal and state laws [6,7]. All 50 US states have legislated against the possession, manufacture, distribution, transportation, importation and exportation of these substances. Since not all members of a given fungal genus contain psilocin or psilocybin, identification of an unknown fungal sample to the species level is required for legal proceedings.

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To determine the species of an isolate, among the hallucinogenic fungi, requires the analysis of several morphological characteristics. Forensic samples of these fungi are usually dried or powdered and, as such, the morphological characteristics are obscured. A DNA-based identification procedure would allow the identification of samples in the absence of morphological characteristics. The development of an identification procedure that will differentiate illegal from legal mushrooms requires a region of known DNA sequence variability. Many fungal taxonomy studies have used DNA sequence of the non-coding internal transcribed spacer (ITS) regions (locus 1 and 2) of the nuclear ribosomal RNA repeat for resolving relationships at the genus and species levels [8–12]. In these studies, oligonucleotide primers for PCR amplification are designed to hybridize to the conserved regions of this otherwise highly variable locus. After amplification, the sequence of the PCR product is determined. This region was previously used by Lee et al. [4] to differentiate illicit fungi of the *Psilocybe* and *Panaeolus* genera found in Scotland and the ITS-based identifications appeared superior to those based on multi-locus analysis [13,14]. We examined the use of the ITS-1 region for identification of hallucinogen containing species in North America with the idea of confirming Lee et al. [4] findings and extending them to North America with a substantially larger set of isolates. In fungal taxonomy research, ambiguities in phylogenetic analysis using the ITS-1 make species level discrimination difficult [15–17]. Resolution of phylogenies in these instances is often possible using the nuclear large subunit of rRNA (nLSU, 28S). This region is less variable than the ITS-1 region and is readily amplified from a large group of basidiomycete fungi [2,18–21]. In this study we use a standard phylogenetic analysis of nucleotide sequences from ITS-1 region and determine these sequences do not allow adequate discrimination of species. We select a subset of the species unresolved by the ITS sequence to determine if the nLSU rRNA sequences provide better phylogenetic signal. The resulting nLSU-based trees clearly separate fungal species containing hallucinogens from those that do not. This separation of branches in the tree is statistically well supported and leads us to propose a phylogenetic approach for the forensic identification of illicit fungi.

2. Materials and methods

2.1. Fungal cultures and strains

Under Health Canada Permit 9109-08-00, fungal cultures were received on Difco Bacto Malt Extract slants from the Canadian Collection of Fungal Cultures (CCFC). At this branch of Agriculture and Agri-Food Canada, cultures have been collected, identified based on morphology and maintained as a reference collection (http://sis.agr.gc.ca/brd/ccc/cccintro.html). We selected 33 isolates representing seven genera. These include; hallucinogen containing genera (*Psilocybe*, *Panaeolus*, *Gymnopilus*) and closely related non-hallucinogen containing genera (*Stropharia*, *Melanotus*, *Hypholoma*) as well as the commonly available white button mushroom, *Agaricus bisporis*. A list of cultures and their collection sites is presented in Table 1. Not included in the table are two ‘‘unknown’’ isolates, labeled H4 and P1 that were received as mycelia on 2% Malt Extract agar medium. The ‘‘unknown’’ isolates were identified independent of this study by a mycologist at the University of Toronto. Both ‘‘unknown’’ isolates were analyzed with the other fungal isolates, and used as an independent test of our experimental approach.

2.2. Growth and culturing

Excised blocks of fungal mycelia were transferred from cultures growing on slants into sterile Petri dishes, cut into small pieces (less than 0.5 mm) using a sterile scalpel and inoculated into liquid CYM (0.46 g KH_2PO_4, 1.0 g $K_2HPO_4{\cdot}3H_20$, 0.5 g $MgSO_4{\cdot}7H_20$, 20.9 g D-glucose, 2.0 g of Difco Yeast Extract and 2.0 g of Difco Bacto Peptone per liter). The cultures were then grown at room temperature for 15 days or until mycelia covered the plate. Mycelia were harvested by filtration through cheesecloth and rinsed with double distilled water. Wet mycelia was then used directly for DNA isolations following Protocol 2 or freeze-dried and stored at −20 °C for future DNA isolations using Protocol 1 or 2 (Section 2.3).

2.3. DNA extraction

We tested two genomic DNA extraction methods. Protocol 1: Smith et al. [22] revision of the Zolan and Pikkula's CTAB extraction method [23]. Protocol 2: the Promega Wizard Genomic DNA Purification Kit, used following the manufacturer's suggested protocol. Integrity of genomic DNA was assessed by agarose gel electrophoresis. Both methods produced comparable results (data not shown) although DNA was isolated in shorter time with the Wizard Kit.

2.4. DNA amplification

Amplification of the ITS-1 locus was performed using primers ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) as described by White et al. [8]. Amplification of a portion of the nLSU was performed using primers L7 (5′-TACTACCACCAAGATCT-3′) and 5.8SR (5′-TCGATGAAGAACGCAGCG-3′) as described by Vilgalys [24,25]. Reaction volume was 20 ul containing: genomic DNA, 1× buffer (50 mM KCl, 10 mM Tris–HCl pH 9, 0.1% Triton X-100), 0.2 μM dNTPs, 2 mM $MgCl_2$, 0.5 units Taq DNA Polymerase (Promega) and 0.5 μM of each primer. Amplifications were carried out in an Applied Biosystems 96-Well

Table 1
Isolates used in this study

Cultures	DAOM[a]	Hallucinogenic[b]	Collection site[a]	GenBank accession numbers
Psilocybe				
Australiana	198379	Yes	California	AY129366, AY129382
Caerulipes	170821	Yes	Gatineau Park, QC	AY129371
Crobula	169914	No	Kouchibouguac, NB	AY129359
	182433	No	Point Pelee, ON	AY129358
Cubensis	169061	Yes	University of British Columbia	AY129351, AY129376
	188193	Yes	Health Canada	AY129342, AY129377
Merdaria	180420	No	Vancouver Island, BC	AY129372, AY129388
Montana	167409	No	Mer Bleu, ON	AY129360, AY129386
	197493	No	Canis Bay Township, ON	AY129352, AY129387
Pelliculosa	226106	Yes	Charlotte Islands, BC	AY129354
Quebecensis	187850	Yes	Gros Morne National Park, NF	AY129373, AY129381
Semilanceata	170014	Yes	New Brunswick	AY129349, AY129378
	178932	Yes	British Columbia	AY129353, AY129380
	226115	Yes	Charlotte Islands, BC	AY129350, AY129379
Silvatica	187832	Yes	Gros Morne National Park, NF	AY129362, AY129383
Panaeolus				
Sphinctrinus	180389	Yes	Norgate Lookout, MB	AY129348, AY129385
Uliginosus	176594	Yes	Whirlpool Lake, MB	AY129363, AY129384
Gymnopilus				
Liquiritae	149442	Yes	Vancouver Island, BC	AY129367
	169049	Yes	Lac Nichabeau, QC	AY129343
	169050	Yes	Lac Nichabeau, QC	AY129344
Luteofolia	46882	Yes	Cantley, QC	AY129370
	198667	Yes	Aylmer, QC	AY129345
Spectabilis	180876	Yes	Cantley, QC	AY129346
Hypholoma				
Capnoides	189456	No	Cantley, QC	AY129341
Sublateritium	54132	No	Gatineau Park, QC	AY129340
	197488	No	Kanata, ON	AY129347
Tuberosum	191771		Vancouver, BC	AY129369
Caricicola	187555	No	Naikon, BC	AY129365
Hartii	160088	No	Kirkland Lake, ON	AY129364
Stropharia				
Rugosoannulata	187501	No	Beausejour, MB	AY129361
Semiglobata	187181	No	Algonquin Park, ON	AY129368
Agaricus				
Bisporus	I_3	No	Israel	AY129375, AY129390
	Ag50	No	California	AY129374, AY129389

[a] Department of Agriculture, Ottawa, Mycology (DAOM). Herbarium specimens are numbered and accessioned as part of the Canadian Collection of Fungal Cultures.
[b] Obtained from [30–36].

GeneAmp 9700 thermal cycler at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min for 35 cycles. Fragment size and number was assessed by agarose gel electrophoresis. If contaminating bands were detected the predicted, major PCR amplified product was gel purified to ensure clean sequence determination. PCR products were purified using the QIAgen PCR Purification and Gel Extraction Kits following the manufacturer's suggested protocols.

2.5. DNA sequencing

Purified PCR products were sequenced using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, v. 3.0 (Applied Biosystems) and reaction products were separated and analyzed on the ABI Prism 310 and/or the ABI Prism 3100 Genetic Analyzers. Amplification primers ITS2 and ITS5 [8] were used for sequencing of the ITS-1 locus and primers LR5 (5′-TCCTGAGGGAAACTTCG-3′), LR16 (5′-TTCCACCCAAACACTCG-3′), LR0R (5′-ACCCGCTGAACTTAAGC-3′), and LR3R (5′-GTCTTGAAACACGGACC-3′) [24,25] were used for sequencing of the nLSU rRNA region. The sequences of the ITS and nLSU regions were determined using populations of amplified products from two or more independent amplifications. Sequence data were submitted to GenBank (http://www.ncbi.nlm.nih.gov/) and were registered with the accession numbers AY129340 through AY129390 (Table 1).

2.6. Phylogenetic and data analysis

Electropherograms were manually edited, contigs were assembled and multiple alignments were created for all sequence data using Genetool software (Biotools Inc). A neighbor-joining (N-J) distance algorithm using the Kimura-2 parameter model as implemented by PAUP (v.4.0b10) [26] was used for phylogenetic analysis. Heuristic analysis using parsimony was also utilized. Two isolates of the distantly related *Agaricus bisporus* (common button mushroom), obtained from P. Horgen (University of Toronto at Mississauga), were used as outgroups in both analyses (Table 1).

2.7. Identification of genus-specific DNA sequences

All ITS-1 locus sequence data were aligned to each other and screened for the *Psilocybe* specific (Table 2) and *Panaeolus* specific regions as was previously described [4].

3. Results

3.1. Amplification of ITS-1 locus and the 5′ portion of the nLSU rRNA region

The ITS2 and ITS5 primers successfully amplified the ITS-1 locus from fifteen *Psilocybe*, two *Panaeolus*, six *Gymnopilus*, two *Melanotus*, two *Stropharia*, four *Hypholoma* and two *Agaricus* isolates (33 total, Table 1). Amplified fragment size varied from 300 to 350 bp as determined by agarose gel electrophoresis and confirmed by DNA sequencing. This variation in fragment size reflects variability in nucleotide sequence (see Section 3.3).

The LR7 and 5.8SR primers [24,25] were used to amplify the 5′ portion of the nLSU rRNA region, which includes three divergent or 'D' domains [27,28]. Amplifications were carried out on eleven *Psilocybe*, two *Panaeolus* and two *Agaricus* isolates. This group included the outgroup and key species within the genera containing the illicit species as well as those species for which we had multiple isolates and the species that were not resolved using the ITS-1 sequences. In nine of the nLSU-amplified products, a secondary short fragment as well as a major fragment of the expected size was present. The larger fragment was gel extracted prior to sequencing.

3.2. Sequence data from the ITS-1 locus and the 5′ portion of the nLSU rRNA region

Both strands of the PCR amplified product from the ITS-1 locus were sequenced. Investigation of aligned sequences reveals over 100 of the 452 character positions has base pair substitutions, insertions or deletions. A previous study [4] used the ITS-1 region to develop *Psilocybe* and *Panaeolus* specific primers for European (Scotland) fungal specimens. This identified *Psilocybe* primer sequence is present in the North American *Psilocybe* isolates. However, this sequence is also present in isolates of the genus *Panaeolus* and of the non-hallucinogenic genera *Hypholoma*, *Stropharia*, and *Melanotus* (Table 2). This indicates that these primers would amplify DNA from genera that do not produce the hallucinogenic compounds and are therefore not illegal. The same analysis was also performed for the *Panaeolus* specific primer-binding region [4]. The sequence that would allow amplification using this primer is found in only one of the *Panaeolus* isolates we investigated (data not shown).

Both strands of the PCR amplified product from the nLSU rRNA region were sequenced using primers LR5, LR16, LR0R, and LR3R [24,25]. Alignment of the nLSU sequences identified 50–60 nucleotide deletions, insertions and substitutions in the 1149 character region. This is less variation than that observed for the ITS-1 data. The alignment of nLSU DNA sequences was examined for nucleotide differences between hallucinogen containing and non-hallucinogen containing isolates. Two regions of variation were detected (Section 3.5). Sequencing reactions were repeated for all isolates and as such the final sequences were confirmed from more than one population of PCR products.

3.3. Phylogenetic analysis

Nucleotide sequences were aligned and trees were constructed utilizing the neighbour-joining distance algorithm

Table 2
Aligned sequences from the ITS-1 locus showing the region proposed to be *Psilocybe* specific

Isolate	'Psilocybe specific' region	Illegal
Psilocybe specific region [4]	ATATCTCCACCTGTGCACCTTT	
Ps. cubensis-188193	ATATTTCCACCTGTGCACTTTT	Yes
Ps. cubensis-169061	ATATTTCCACCTGTGCACCTTT	Yes
"Unknown"-H4	ATATTTCCACCTGTGCACTTTT	Yes
"Unknown"-P1	ATATTTCCACCTGTGCACTTTT	Yes
Ps. semilanceata-170014	ATCTCTCCACCTGTGCACCTTT	Yes
Ps. semilanceata-226115	ATCTCTCCACCTGTGCACCTTT	Yes
Ps. semilanceata-178932	ATCTCTCCACCTGTGCACCTTT	Yes
Ps. quebecensis-187850	ATATCTCCACCTGTGCACCCTT	Yes
Ps. silvatica-187832	ATCTCTCCACCTGTGCA-GTTT	Yes
Ps. pelliculosa-226106	ATCTCTCCACCTGTGCACCTTT	Yes
Ps. australiana-198379	ATATTTCCACCTGTGCACCTTT	Yes
Ps. caerulipes-170821	ATATTTCCACCTGTGCACCTTT	Yes
Ps. merdaria-180420	ATATCTCCACCTGTGCACC-TT	No
Ps. crobula-182433	ATATTTCCACCTGTGCACCTTT	No
Ps. crobula-169914	ATATTTCCACCTGTGCACCTTT	No
Ps. montana-197493	ATATTTCCACCTGTGCACCTTT	No
Ps. montana-167409	ATATTTCCACCTGTGCACTTTT	No
H. sublateritium-197488	ATATCTCCACCTGTGCACCTTT	No
H. sublateritium-54132	ATATCTCCACCTGTGCACCTTT	No
H. capnoides-189456	ATATCTCCACCTGTGCACCTTT	No
H. tuberosum-191771	ATATCTCCACCTGTGCACCTTT	No
S. rugosoannulata-187501	ATCTCTCCACCTGTGCACCTTT	No
S. semiglobata-187181	ATATCTCCACCTGTGCACCTTT	No
M. hartii-160088	TTGTTTTTCCACCTGTGAACACACT	No
M. caricicola-187555	ATATTTCCACCTGTGCACCTTT	No

Shaded characters represent nucleotide variations with respect to the reference sequence.

for the ITS-1 and the 5′ portion of the nLSU rRNA. Similar trees were generated using Parsimony analysis, however the N-J algorithm has the advantage of being less computationally intensive and of using a base-change model that assumes variable rates of nucleotide substitutions [29]. The N-J trees were analyzed for our datasets. Based on the ITS-1 sequences, there are discrepancies between the phylogenetic tree and the morphological identification of some reference isolates (Fig. 1). Notably, the two isolates of *Psilocybe montana* are in separate branches as are the two isolates of *Psilocybe cubensis*. To determine if nLSU data is more consistent with morphological identifications, a N-J cladogram based on a dataset comprised of eleven *Psilocybe*, two *Panaeolus* and two *Agaricus* isolates was generated. With the nLSU sequences, *Psilocybe montana* isolates group in a single clade as do *Psilocybe cubensis* isolates (Fig. 2). We also observed that the nLSU data clearly separates hallucinogenic from non-hallucinogenic isolates. The three non-hallucinogenic isolates (two *Psilocybe montana* and one *Psilocybe merdaria*) branch to a separate clade with 80% bootstrap support. To rule out the possibility of bias in the subset of isolates used for the nLSU cladogram, a tree based on the same isolates was generated from the ITS-1 data. The ITS tree topology is inconsistent with morphological data (Fig. 3).

3.4. Sequence-based identification of hallucinogenic species

Visual inspection identified a short sequence containing two subregions that varied between the isolates that contain hallucinogens and those that do not (Fig. 4). Further analysis revealed that this region does not contain a contiguous stretch of nucleotides that would allow the design of a primer pair specific to illegal mushrooms. Instead, we propose the use of sequence data that encompasses the divergent D1–D3 domains, found within the first 900 bp of the nLSU gene, for identification. Comparative analysis to a reference database of sequences from this region would be used for identification of the "unknown" samples.

3.5. Experimental test with two 'street' samples

As a test of this comparative sequence approach, we analyzed two "unknown" fungal samples, H4 and P1, determined by independent morphological analysis to be samples of *Psilocybe cubensis*. The DNA sequence of the ITS-1 and the first 900 bp of the nLSU were determined. As part of the initial investigation, both samples were screened for the '*Psilocybe* specific' region [4]. This region was found for both isolates, however base substitutions were apparent

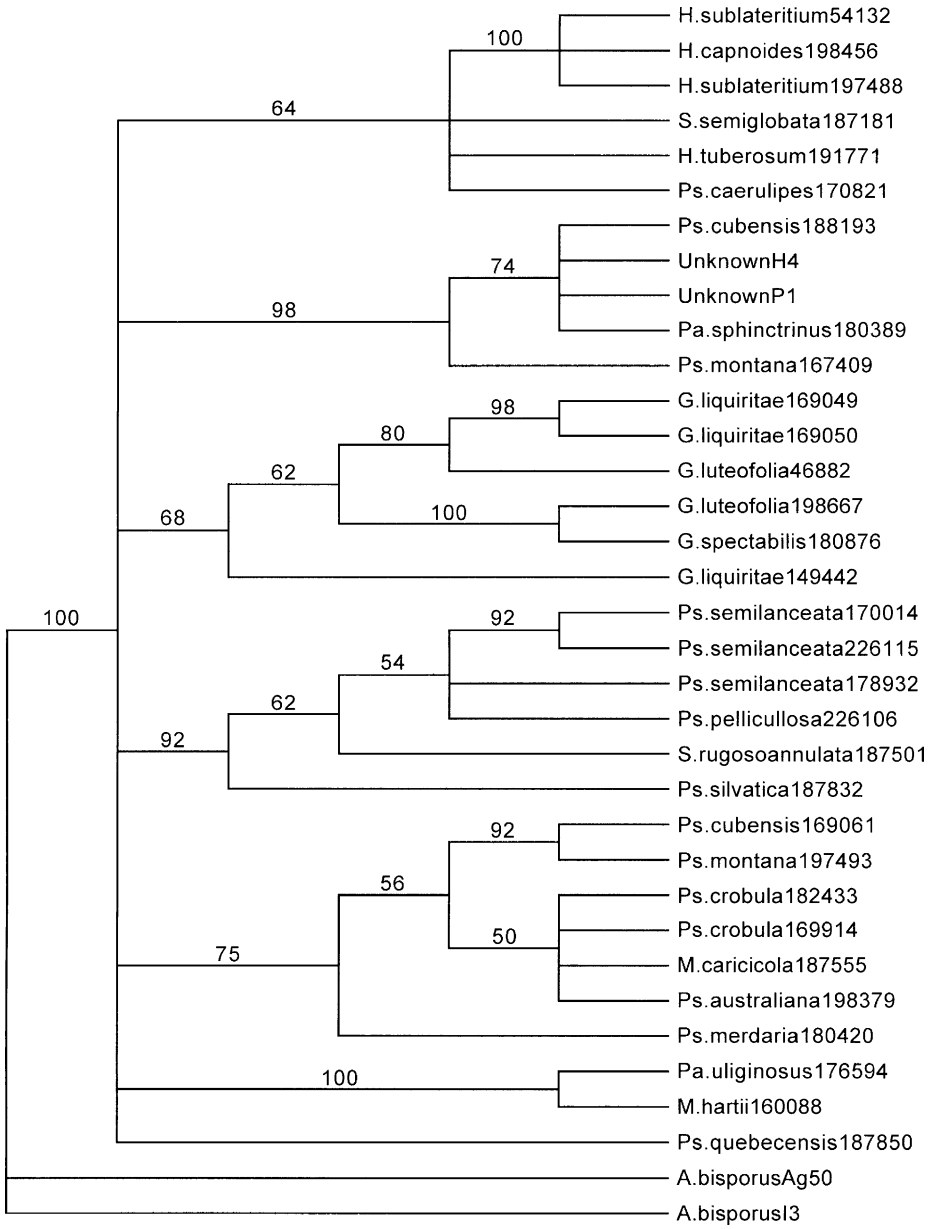

Fig. 1. Phylogenetic tree constructed using the neighbour-joining distance algorithm and the Kimura-2 parameter model. Analysis was based on 35 aligned sequences from the ITS-1 locus. Numbers indicate percent bootstrap support (1000 replications) where values greater than 50% are indicated on the branches.

(Table 2). A cladogram based on the ITS-1 sequence data of H4 and P1 was generated and shows that both samples cluster with isolates of *Psilocybe cubensis*, *Panaeolus sphinctrinus* and *Psilocybe montana* (Fig. 3). This tree topology does not allow us to determine if H4 and P1 produce hallucinogens. In contrast, the cladogram constructed from the nLSU sequence has H4 and P1 grouped with isolates of the hallucinogenic *Psilocybe cubensis* in a single clade with 95% bootstrap support (Fig. 2).

4. Discussion

All DNA-based identifications require variability in DNA sequence. The level of variation uncovered often depends on the sample size studied and the region of the genome analyzed. In illicit mushroom identification, attempts to determine whole genome variation through the use of AFLP and RAPD analyses lack resolution [13,14]. In identifications based upon sequence differences in a small region of

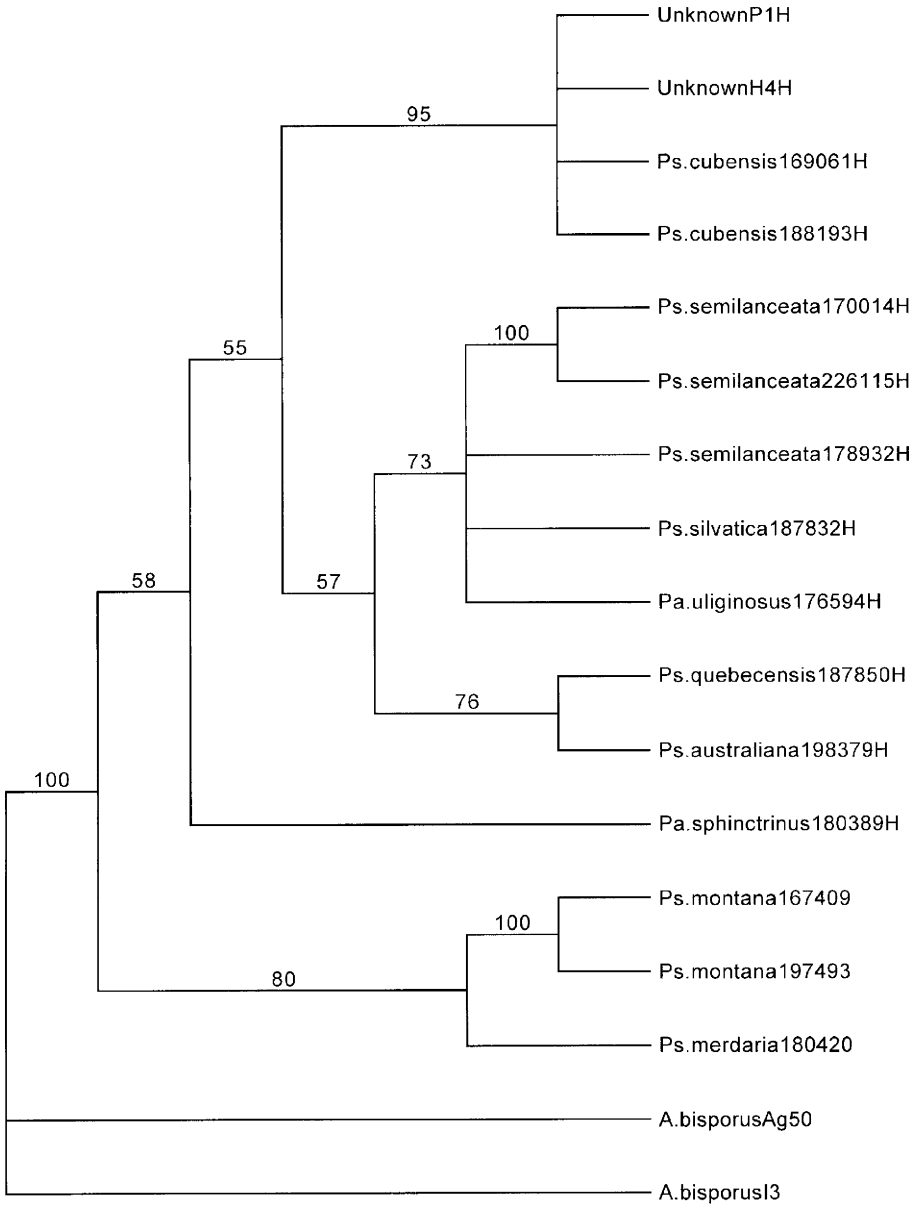

Fig. 2. Phylogenetic tree constructed using the neighbour-joining distance algorithm and the Kimura-2 parameter model. Analysis was based on 17 aligned sequences from the nLSU rRNA region. Numbers indicate percent bootstrap support (1000 replications) where values greater than 50% are indicated on the branches. Isolates labeled H4 and P1 were "unknown" samples used as a test of our experimental approach. The 'H' following a taxon name indicates the isolate contains the controlled substances (hallucinogenic).

the genome, the ITS-1 locus, Lee et al. [4] were able to design primers to specifically amplify two key genera of fungi from Scotland containing psychoactive compounds. In this study we set out to extend the use of ITS sequences to the identification of illicit fungal species in North America. We found that the sequences used for primer design in the Scottish study were not specific to the corresponding genera in the North American samples. We further noted that identifying fungi to genera is not sufficient in a forensic context, because some genera contain species that produce psychoactive compounds and species that do not. We attempted, using all variable characters in the ITS sequence, a phylogenetic approach for fungal identification to the level of species. Our findings determined that the ITS sequences lacked the type of variation required for this level of resolution, but that the phylogenetic approach had promise as an identification tool. To verify that the approach was appropriate for fungal identification in this context we

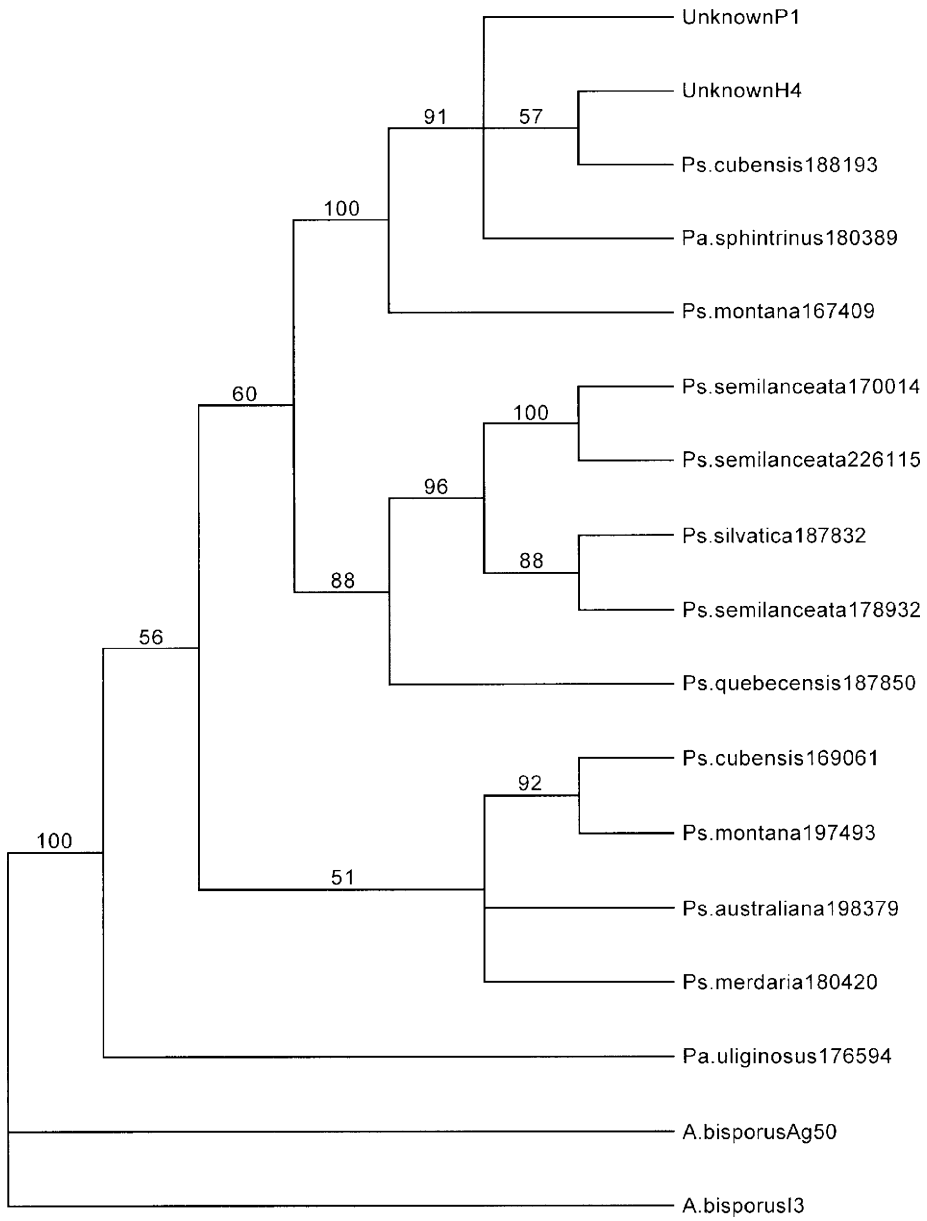

Fig. 3. Phylogenetic tree constructed using the neighbour-joining distance algorithm and the Kimura-2 parameter model. Analysis was based on 17 aligned sequences from the ITS-1 locus. Numbers indicate percent bootstrap support (1000 replications) where values greater than 50% are indicated on the branches. Isolates labeled H4 and P1 were "unknown" samples used as a test of our experimental approach.

assessed the use of sequences from the nLSU of the rRNA. We focused on those species and isolates that were not well separated in the ITS investigation, selecting 33 North American isolates representing 23 species and containing multiple isolates of the same species; hallucinogen producing species, and morphologically similar non-producing species. This provides a set of isolates that allow the determination of specificity of an identification protocol. Previous research [4] did not incorporate these criteria into their analysis.

The approach we use focuses on the direct assessment of variation by sequencing the DNA. Others have used indirect means to infer the underlying sequence variation [4,13,14]. These are primarily based upon different fragment lengths among species resulting from PCR amplification or restriction digest. This method of detecting variation includes, but does not distinguish between sequence changes, insertions and deletions. The use of multi-locus analysis techniques such as AFLP and RAPD proved to be ambiguous and not

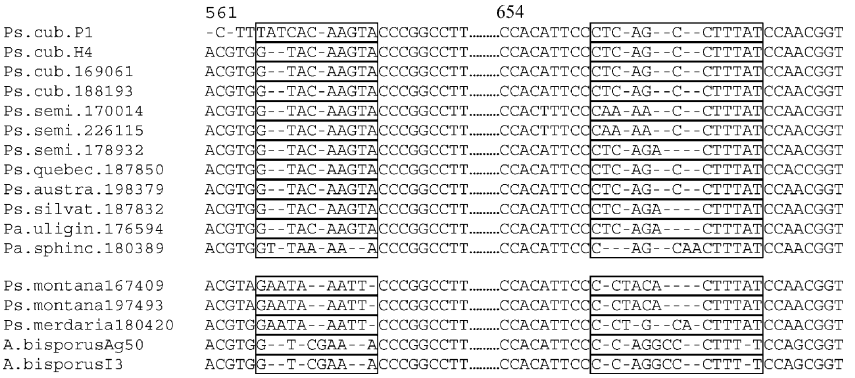

Fig. 4. Regions of variability found within the nLSU rRNA sequences. The first twelve sequences represent hallucinogenic species and the last five sequences represent non-hallucinogenic species. Nucleotide differences distinguish hallucinogen containing from non-hallucinogen containing isolates.

amenable to laboratory comparisons or the production of fragment pattern databases [21,22]. The direct assessment of sequence variation may allow unambiguous identification of fungal isolates.

To determine if sequence variation in the ITS-1 locus has discriminating power we compiled sequence data for all cultures and a cladogram was generated (Fig. 1). We included multiple isolates of *Psilocybe montana* and *Psilocybe cubensis* in this analysis. If the sequence of the ITS had appropriate resolving power we would expect that multiple isolates of a given species would appear on the same terminal branches. This is not the result we obtained, instead the topology generated illustrates that morphologically defined species are not supported by the ITS-1 sequence data (Figs. 1 and 3). The lack of support for morphological identification limits the use of the ITS-1 sequence data for forensic purposes. These results contrast those of Lee et al. [4] who proposed the use of the ITS-1 locus in distinguishing illicit fungal genera and designed an amplification primer to rapidly assess this. However, controlled substances are present in some species of a genus, but not others. This indicates a need for species level identification and our data suggest that the ITS-1 locus is inappropriate for this level of resolution. Fungal taxonomic studies have also demonstrated that ITS-1 data may be ambiguous in discriminating certain taxonomic levels [15–17]. It may be possible to use the ITS-1 region in a taxon-based identification system, if it was combined with another variable region(s). We assessed this possibility by carrying out a phylogenetic comparison with the combined ITS-1 and nLSU sequences. The combined sequence data produced poorly resolved cladograms (data not shown). This indicates that the ITS sequences do not allow the discrimination of species even if combined with another variable sequence.

The divergent 'D' domains of the nLSU rRNA have been used in fungal systematic studies and sequence data from these variable regions have produced well-resolved phylogenetic trees [2,18–21,24,25]. A subset of fungal isolates was chosen to investigate whether sequence based on this region would produce cladograms that resolved fungal species and separated hallucinogenic and non-hallucinogenic *Psilocybe* species. We were able to amplify the nLSU rRNA region from all species assessed, however, the isolates used for sequence determination were those informative species that were not differentiated in the ITS investigation. With the nLSU rRNA sequence data, we observed clustering of the *Psilocybe cubensis* isolates as well as the *Psilocybe montana* isolates (Fig. 2). Most significant however, is the ability of the nLSU sequence data to separate hallucinogenic from non-hallucinogenic isolates with 80% bootstrap support (Fig. 2). A bootstrap analysis is a statistical test of the position of an isolate or group of isolates in the tree. It is based upon repeated derivations (1000 in our test) of the tree from the input sequence data. A higher percentage represents increased confidence in the clustering or separation. The well-supported topology separating hallucinogenic from non-hallucinogenic species meets the forensic requirement for clear distinction. Biologically this separation is interesting because it suggests a linkage between speciation and retention of the ability to produce psychoactive compounds. An independent study investigating basidiomycete taxonomy supports this clear separation (Moncalvo personal communication) and validates the use of the nLSU sequence for future, more extensive, analysis of this group of fungi at a population and species levels.

The multicopy nature of the LSU RNA makes it a robust target for amplification, but raises the question of sequence variability within an individual that may obscure identification. Independent tests in our laboratory and others have shown that if a sequence variant is 10% or less of the number of copies of the dominant sequence type it will not be detected. As a result the population of fragments amplified for each sequence determination carried out here represent the dominant sequence type. The sequence for each region was determined from populations of amplified fragments from at least two separate amplifications. This allows us to

assess variation in the highest copy sequence and, while potentially missing variation of lower copy number sequences; this provides a reproducible method of species identification. In our laboratory three or more amplifications of these regions from fungal cultures did not add further information for species identification to that obtained from two amplifications.

Visual inspection of the nLSU sequence data revealed sufficient nucleotide variation in two stretches of sequence that would allow us to distinguish species that produce the illegal compounds from similar species that do not (Fig. 4). Unfortunately the uneven spacing of nucleotide variation prevented the design of a specific primer pair to selectively amplify the nLSU DNA region of hallucinogenic fungi. Nonetheless, differences are apparent between the nLSU sequences from illegal and legal fungal isolates. To capture the discriminating information in this sequence variation, we propose establishing a fungal sequence library of the nLSU rDNA and the use of this library for comparison to ''unknown'' samples using a standard phylogenetic approach. This would allow: (1) global comparisons of cultures, a concern considering how spores of magic mushroom are internationally sold and (2) the standardization based upon existing commercially available kits and software specifically designed for such a use (for example, the MicroSeq D2: LSU rDNA Sequencing Kit from Applied Biosystems).

As an experimental test of our approach, we analyzed two ''unknown'' fungal samples labeled H4 and P1. A cladogram based on the ITS-1 data grouped both ''unknown'' samples with isolates of *Psilocybe cubensis*, *Psilocybe montana* and *Panaeolus sphinctrinus* (Fig. 3). This cladogram does not separate species that contain hallucinogens from those that do not. When based on the nLSU data however, we observe tight clustering of both ''unknown'' samples with the two known *Psilocybe cubensis* isolates (Fig. 2) indicating that both samples belong to a group of fungi known to produce hallucinogenic compounds and that they are likely isolates of *Psilocybe cubensis* which is consistent with an independent identification based on morphological characters.

Fungal samples seized during forensic investigations are often in a form that makes morphological identification difficult if not impossible. Since the possession of dried, cultured or degraded forms of these fungal specimens is illegal, there exists a need for reliable and automated identification methods. Pending more information on population level variation in each of the species sampled here, phylogenetic analysis of nucleotide sequence variation could be used to precisely identify unknown fungal isolates. The technology currently existing in forensic laboratories can detect this sequence variation. Therefore, the direct assessment of sequence variation of well-characterized genomic regions is a practical and accurate means of unambiguous identification of unknown forensic samples. Our data shows that combining analyses of the ITS-1 and nLSU sequence variation has the resolving power to distinguish very closely related species. We propose that a sequence database of the ITS and the first 900 bp of the divergent nLSU region would provide a reliable and automated approach to the identification of hallucinogen containing fungal isolates. In this approach DNA sequences from unknown fungal samples would be compared to a library of sequences to determine if the unknown isolate is a species that contains controlled substances. The fine resolution provided by sequence analysis suggests that it may also be used in the identification of a wide range of illicit material found in forensic investigations.

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