JOURNAL OF FORENSIC SCIENCES

J Forensic Sci, July 2011, Vol. 56, No. 4
doi: 10.1111/j.1556-4029.2011.01739.x
Available online at: onlinelibrary.wiley.com

TECHNICAL NOTE

CRIMINALISTICS; TOXICOLOGY

Kathryn A. Harper,[1] M.S.; Christine D. Smart,[2] Ph.D.; and R. Michael Davis,[1] Ph.D.*

Development of a DNA-Based Macroarray for the Detection and Identification of *Amanita* Species

ABSTRACT: A DNA-based macroarray was designed to quickly and accurately identify certain *Amanita* mushroom specimens at the species level. The macroarray included probes for *Amanita phalloides* and *Amanita ocreata*, toxic species responsible for most mushroom poisonings, and *Amanita lanei* and *Amanita velosa*, edible species sometimes confused with toxic species, based on sequences of the highly variable internal transcribed spacer (ITS) region of rDNA. A cryptic species related to *A. ocreata* and one related to *A. lanei*, identifiable by ITS sequences, were also included. Specific multiple oligonucleotide probes were spotted onto nylon membranes and the optimal hybridization temperatures were determined. The *Amanita* DNA array was highly specific, sensitive (0.5 ng DNA/μL and higher were detected), and reproducible. In two case studies, the method proved useful when only small amounts of mushroom tissue remained after a suspected poisoning. An identification could be completed in 12 h.

KEYWORDS: forensic science, mushroom poisoning, *Amanita,* oligonucleotide array sequence analysis, DNA, ribosomal spacer, amanitin

Recorded mushroom poisonings date back to 430 B.C. with the death of the wife and three children of the Greek poet Euripides and to the alleged mushroom poisoning of Roman Emperor Claudius by his wife in 54 A.D. (1,2). In 1918, 31 Polish children died after *Amanita phalloides* was prepared as part of a school meal (3). Mushroom poisonings continue to be a health concern today and they are a fairly common medical emergency (4). According to the California Department of Health Services, the California Poison Control System reported 916 mushroom ingestion cases in 2006 (5). Of the 370 people who sought health care treatment, 16 were admitted to the hospital, and 10 had a "major health outcome," resulting in liver failure, renal failure, or death (5). Nationally, the American Association of Poison Control Centers reported 7733 cases of mushroom exposures in 2007 and death of a 67-year-old woman following ingestion of *A. phalloides* (6). More recently, an 82-year-old man died of liver failure in March of 2009, 7 days after eating what was described as a "heaping plate" of wild mushrooms picked near Santa Barbara, California (7). The mushrooms were reportedly the toxic *A. ocreata*.

Species in the genus *Amanita* are responsible for the vast majority of mushroom poisonings. *Amanita phalloides*, also known as the death cap, is probably responsible for more than 90% of mushroom poisoning fatalities in the U.S. (8,9). However, the destroying angel, *A. ocreata*, is also responsible for a significant number of poisonings in some years. Both *A. phalloides* and *A. ocreata* are widespread, occur commonly in California, and are deadly poisonous (10). These toxic species contain a number of different toxins, most notably the amatoxins, which include α-amanitins, hepatotoxic cyclopeptides responsible for most fatalities. Two additional groups of cyclopeptides, phallotoxins and virotoxins, may also contribute to poisonings from mushroom ingestion. In addition to the genus *Amanita*, some species in four other genera, *Galerina*, *Lepiota* sensu lato, *Cortinarius*, and *Conocybe*, are known to contain hepatotoxic cyclopeptide toxins (1,11,12). In humans, α-amanitins are lethal at around 0.1 mg/kg of body weight; therefore, one mushroom cap can contain a lethal dose (12). On average, species of *A. phalloides* and *A. ocreata* contain 1.5–2.3 mg amanitins per gram of mushroom dry weight (12). α-Amanitin inhibits RNA polymerase II, which causes the shutdown of transcription and therefore prevents subsequent protein synthesis. Severe poisonings by amanitins can lead to liver failure, kidney failure, multi-organ failure, and death (12).

There are four stages associated with lethal or near-lethal cyclopeptide toxicity (1). The first stage, the latent period, can last 6–24 h following mushroom ingestion, during which no clinical symptoms of poisoning are observed. After the latent period, the second stage, lasting 12–24 h, is characterized by severe gastrointestinal distress, which can itself lead to hospitalization owing to dehydration and electrolyte imbalance. The third stage can last 12–24 h and is marked by apparent improvement when, in actuality, liver and sometimes kidney function begin to show signs of failure. Deterioration of the liver, kidneys, and other organs continue in the fourth stage, which can last 4–7 days. Around 20% of survivors of amatoxin ingestion develop chronic hepatitis (1). While numerous treatments for cyclopeptide toxicity have been explored, none has been clinically proven and current medically accepted treatment is described as supportive. A liver transplant may offer the only option for those with severe, life-threatening liver damage (1).

[1]Department of Plant Pathology, University of California, Davis, CA.
[2]Department of Plant Pathology, Cornell University, 630 West North Street, Geneva, NY.
*Present Address: Counterterrorism Forensic Science Research Unit, FBI Academy, Building 12, Quantico, VA.

Received 1 Feb. 2010; and in revised form 15 May 2010; accepted 5 June 2010.

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Numerous scenarios can lead to toxic mushroom ingestion. The most common appear to be associated with mushroom misidentifications, which can lead to the consumption of a large quantity and lethal dose of toxic mushrooms. Mushroom hunters who migrate from other countries represent a high-risk group as they are more likely to mistake a toxic species for a morphologically similar edible species from their native region. Exposures involving accidental ingestion of mushrooms by children and pets are not uncommon.

While mushroom foraging can be a fun and exciting activity, it comes with an inherent risk. It is critically important that mushroom hunters recognize the associated risk and take the time to positively identify each mushroom picked for the table. There are numerous myths concerning fool-proof ways of discerning edible from poisonous mushroom specimens, but there is no substitute for field experience. General mushroom identification is currently performed by experts in mycology (mycologists) and is based on fruiting body morphology, spore characteristics, the location source and proximity to certain species of trees, and, if necessary, DNA-based methods. The highly variable internal transcribed spacer (ITS) region of the nuclear ribosomal DNA is often used to identify fungi at the species level (13). In situations involving a suspected mushroom poisoning, it is crucial to accurately identify the fungal species involved as quickly as possible so that appropriate medical treatment can be administered if necessary. If all the mushrooms are not consumed and an intact specimen remains, it is sometimes possible to identify the mushroom based on morphological features. However, it is not always the case that an identifiable specimen is available following mushroom consumption. A sample from uncooked mushroom debris, stomach contents, or a cooked dish (e.g., spaghetti sauce) is typically impossible to identify based on physical characteristics. In these cases, DNA-based identification tests can still be used. To compare DNA sequences of an unidentified specimen with available ITS sequences in GenBank or a personal database, the DNA of such a sample needs to be extracted, amplified, and sequenced. Given the time constraints of a mushroom poisoning case, it may not be possible to accomplish this testing in sufficient time. To speed the identification of unrecognizable or obliterated mushroom samples, we developed a DNA-based macroarray. The macroarray, which is conceptually equivalent to a microarray, involves the use of short oligonucleotide probes fixed to nylon membranes as the hybridization medium. To design the probes, species-specific areas of the ITS region were identified for five *Amanita* species: *A. phalloides*, *A. ocreata*, a cryptic species related to *A. ocreata* and hereafter called *Amanita* affinity (aff.) *ocreata*, *Amanita lanei* (also known as *Amanita calyptrata*), *Amanita velosa*, and an undescribed species related to *A. lanei*, hereafter called *A.* aff. *lanei*. The latter species is a yellow-capped mushroom that fruits in the spring in the Sierra Nevada foothills. These species were chosen because they are either toxic or morphologically very similar to toxic species. Mushrooms belonging to the genus *Amanita* are often not recommended for the table because of the potentially deadly consequences of eating a toxic "look-alike." However, *A. lanei*, *A.* aff. *lanei*, and *A. velosa* are all edible species and commonly eaten by mushroom hunters in California. These and *A. phalloides* and *A. ocreata* represent those likely to be involved in misidentification and poisoning cases.

Materials and Methods

Specimen Collection

Mushroom specimens were collected in tanoak (*Lithocarpus densiflorus*), Douglas-fir (*Pseudotsuga menziesii*), and Bishop pine (*Pinus muricata*) mixed forests or under coastal live oak (*Quercus agrifolia*) in central and northern California. Tissue was removed from the center of the stipe (stalk) of each mushroom and either processed immediately or frozen at −20°C in 15-mL plastic conical tubes. The pileus (cap) of each mushroom was cut off the stipe and placed lamellae (gill)-side down on a white index card to obtain a spore print. The stipes and caps were finally dried in a laboratory oven at 60°C for 1–2 days, depending on the size of the specimen. Once dried, each specimen and corresponding spore print were stored in a labeled specimen box at room temperature. In addition to the mushrooms collected by laboratory personnel, we used several specimens sent to our laboratory for identification.

α-Amanitin Testing

Several specimens were tested for the presence of α-amanitin using liquid chromatography/mass spectrometry (LC/MS) by the California Animal Health and Food Safety (CAHFS) Laboratory System at UC Davis. The method used to detect α-amanitin in serum and liver was adapted for the detection of the toxin in fungi (11).

DNA Extraction, Amplification, and Sequencing

Mushroom DNA was extracted from frozen, fresh, or dried tissue using Qiagen's silica gel membrane DNeasy Plant Mini kit (Valencia, CA) according to the manufacturer's protocols, with one exception: 400 μL of a mixture of mercaptoethanol and cetyltrimethylammonium bromide (CTAB) buffer (10 μL mercaptoethanol in 2 mL CTAB [50 mL 1 M Tris–HCl pH 8, 50 mL 0.5 M EDTA, 40.9 g NaCl, 5 g PVP-40, and 12.5 g CTAB, brought up to 500 mL with water]) was added to each sample in the place of Qiagen's Buffer AP1. Approximately 50 mg of tissue was ground in liquid nitrogen using a mortar and pestle prior to the start of DNA extraction.

Following DNA extraction, the ITS region of the nuclear rDNA was amplified by polymerase chain reaction (PCR) using primers ITS1-F (5′ CTT GGT CAT TTA GAG GAA GTA A 3′) and ITS4-B (5′ CAG GAG ACT TGT ACA CGG TCC AG 3′), which are specific for basidiomycetes (14). DNA was amplified in 50-μL reactions consisting of 27.6 μL sterile water, 5 μL $MgCl_2$, 10 μL 5× Mg Free Buffer, 4 μL 2.5 mM dNTPs, 0.2 μL Promega Go*Taq*, (Promega Corporation, Madison, WI) 0.1 μL 50 μM of each primer, and 3 μL of template DNA. The thermocycler program was as follows: 40 cycles 1 min 94°C, 2 min 55°C, 2.5 min 72°C, and a final extension at 72°C for 10 min. Amplicons were stored at 4°C in the thermocycler until they were frozen at −20°C.

To confirm successful DNA amplification, the PCR product was mixed with Promega Blue/Orange 6× Loading Dye (Madison, WI) and separated by electrophoresis using 1.5 or 2% agarose gels. The gels were then stained with ethidium bromide and visualized under UV light.

Successfully amplified PCR products were purified using Qiagen's QIAquick PCR Purification kit according to the manufacturer's protocol. The purified products were sequenced with primers ITS1-F and ITS4-B at the UC Davis Division of Biological Sciences DNA Sequencing Facility. Sequences were edited and aligned using CLUSTALW (15) in Invitrogen's Vector NTI (16).

Alignment and Probe Design

To accurately visualize unique DNA regions for each target species, we created a sequence alignment that included mushrooms

from this study as well as similar sequences from GenBank (National Center for Biotechnology Information, Bethesda, MD) (Table 1). Following alignment with CLUSTALW in Vector NTI, the alignment was manually adjusted in MacClade (19). To ensure functional probes, we selected oligos 17–27 bases in length with the unique targeted DNA base pair(s) located in the center of the probe sequence (20). In addition, probes were designed to have a melting temperature between 50°C and 60°C (20). The following equation was used to calculate the melting temperature of each probe: $Tm = 64.9 + 41([y + z - 16.4]/[w + x + y + z])$, where $w = A$, $x = T$, $y = C$, and $z = G$ (21). The oligonucleotide probes were synthesized by Sigma Genosys (Sigma-Aldrich Co., The Woodlands, TX).

Numerous species-specific probes were designed for each of the following taxa: *A. phalloides*, *A. ocreata*, *A.* aff. *ocreata*, *A. lanei*, *A.* aff. *lanei*, and *A. velosa*. Separate sets of probes were designed for the two cryptic species of *A. ocreata* because they did not share any unique target regions. Unlike the *A. ocreata* complex, *A. lanei* and *A.* aff. *lanei* shared unique sections within the ITS region; therefore, species-specific probes and probes that targeted both species were designed.

Membrane Spotting

The probes were diluted to 100 μM and mixed 1:1 with 2× spotting buffer to obtain a final probe concentration of 50 μM in 1× spotting buffer (4 μM sodium carbonate buffer, pH 8.4, 3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.01% *N*-lauroyl sarcosine, and 0.004% bromophenol blue). After the probes and buffer were mixed in a skirted 96-well PCR plate, the 50 μM probe/1× spotting buffer solution was spotted onto 8 × 12 cm Amersham Hybond-N^{+} membranes (GE Healthcare, U.K. Ltd., Little Chalfont, Buckinghamshire, U.K.) in quadruplicate using a 96 Pin Replicator and Omni Tray Copier (Nalge Nunc International, Rochester, NY) using V&P Scientific's Pin Cleaning Solution (San Diego, CA) according to the manufacturer's instructions. The membranes were allowed to dry at room temperature for 10 min and then exposed to UV light in an AlphaImager 2200 (Alpha Innotech, San Leandro, CA) to fix the probe/spotting buffer solution to the membranes. The membranes were then washed in 500 mL 0.5% SDS solution at 60°C for 60 min followed by a 5-min wash in 500 mL 1% Tris–HCl (pH 8) solution at room temperature, per manufacturer's protocol. The membranes were wrapped in plastic wrap followed by aluminum foil and stored in the refrigerator at 4°C.

Primers ITS1-F and ITS4-B were spotted alternately along the top and left side of each membrane for positive controls and as a guide for reading the membrane. Hybridization to the positive controls indicated successful macroarray hybridization because ITS1-F and ITS4-B primer sequences are present in all basidiomycete mushrooms. Failure of the positive controls to hybridize indicated a failure in the hybridization process and negated interpretation of any results. Negative controls, which consisted of water and spotting buffer, were included on all membranes.

After the optimum hybridization temperature was determined, the three probes that showed the best hybridization results for each species of interest were selected. The probes were chosen based on species specificity as well as the strength (i.e., darkness) of their resulting hybridization spots. Membranes were then spotted using the newly selected probes and appropriate controls as described previously, i.e., primers ITS1-F and ITS4-B were spotted alternatively along the top and left side of each membrane and served as positive controls. Water and spotting buffer applied to the membranes served as the negative controls. The three strongest probes for each species were spotted in order on the top half of each membrane and in a mixed order on the bottom half for experimental purposes to show that the order and placement of the probes in relation to each other is irrelevant to target binding.

TABLE 1—*Species of mushrooms used in the development of species-specific DNA probes in this study, their GenBank accession numbers of the internal transcribed spacer region (ITS), and their edibility.*

Amanita Species	GenBank ITS Accession No.	Edibility*
Amanita aspera	AF085485	Toxic
Amanita bisporigera	AY550243	Toxic
Amanita caesarea	AY486237	Edible
Amanita constricta	AY228351	Edible
Amanita esculenta	AY436451	Edible
Amanita exitalis	AY436454	Toxic
A. exitalis	AY855212	Toxic
Amanita fulva	AB015692	Edible
Amanita hemibapha	AB015699	Unknown
Amanita jacksonii	AY436461	Edible
Amanita lanei	DQ974693	Edible
Amanita aff. *lanei*	GQ250401	Edible
Amanita longistriata	AB015678	Unknown
Amanita marmorata subsp. *myrtacearum*	AY325826	Unknown
Amanita ocreata	AY918962	Toxic
A. ocreata	GQ250404	Toxic
Amanita aff. *ocreata*	GQ250405	Toxic
Amanita phalloides	EU909444	Toxic
Amanita pseudovaginata	AY436470	Edible
Amanita reidii	AY325824	Unknown
Amanita subjunquillea var. alba	DQ072729	Toxic
Amanita vaginata	AB015691	Edible
Amanita velosa	AY918961	Edible
A. velosa	DQ974692	Edible
A. velosa	GQ250409	Edible
Amanita virosa	AB015676	Toxic
A. virosa	AY325829	Toxic

*Toxicity information determined from references 17,18,22.

Membrane Hybridization

Prior to DNA hybridization on the macroarray membrane, extracted DNA from mushroom specimens was amplified using primers ITS1-F and ITS4-B and purified as described previously. The purified DNA from each specimen was quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE) and adjusted to 10 ng/μL.

The macroarray hybridization was performed using the Amersham Gene Images AlkPhos Direct Labeling and Detection System (GE Healthcare) according to the manufacturer's protocol, with the exception of the hybridization temperature, which was varied between 50°C and 55°C (in 1°C increments). Following a 2-h hybridization in a Labnet Deluxe ProBlot hybridization oven (Labnet International, Inc., Woodbridge, NJ) at rotation speed 2, the membranes were washed twice for 10 min at 50–55°C, followed by two 5-min washes at room temperature (*c.* 25°C). The hybridization and first wash were performed at the same temperature. After the washes, 2 mL of CDP-Star Detection Reagent was applied to each membrane, followed by an incubation of 3–5 min at room temperature. After the excess detection reagent was allowed to drain off, each membrane was wrapped in plastic wrap and placed in an autoradiography cassette facing a sheet of Kodak BioMax Light Film or Denville Scientific HyBlot CL Autoradiography Film (Denville Scientific, Inc., Metuchen, NJ). After the

film was exposed to the membranes for 30 min, it was developed using a Konica SRX-101A Film Processor. (Konica Minolta Medical Imaging USA, Inc., Wayne, NJ)

Mixtures of Mushroom Tissue

To determine whether the macroarray could distinguish individual species in mixed mushroom samples, dried mushroom tissue from selected species was mixed in equal parts (0.01 g each) and DNA was extracted as previously described. The mixtures included the following: *A. phalloides* + *A. lanei*, *A. ocreata* + *A. lanei*, *A. phalloides* + *A. ocreata* + *A. lanei*, and *A. phalloides* + *A. ocreata* + *A. lanei* + *Tricholoma magnivelare*. Following DNA extraction, the macroarray was tested as previously described.

Sensitivity

To determine the threshold of detection for the macroarray, DNA of one specimen of *A. phalloides* with an initial concentration of 40.4 ng/μL and DNA of one specimen of *A. ocreata* with an initial concentration 42.4 ng/μL were diluted to 0 (0%), 0.1 (1%), 0.5 (5%), 1 (10%), 2 (20%), and 4 ng/μL (40%) to test the sensitivity of detection for these species. The dilutions were performed twice for both species.

Specificity

In addition to the trials with DNA samples from target mushrooms, several other species were tested on the macroarray, including *Amanita franchetii* (07040), *Amanita gemmata* (07045), *Amanita muscaria* (07042), *Amanita novinupta* (07063), *A.* aff *pantherina* (08001), *Amanita silvicola* (07061), *A.* sp. (07053), *A.* sp. (07054), *A.* sp (07075), *Agaricus xanthodermus* (09001), *Clitocybe nuda* (08006), *Psilocybe cubensis* (10B), *T. magnivelare* (07064), and *Volvariella speciosa* (08004). DNA and dried tissue from the above specimens are maintained in the R.M. Davis Labaratory, Department of Plant Pathology, UC Davis. The analysis of the macroarray using DNA from nontarget species was performed once, unless cross-hybridization was visualized, in which case that species was tested a second time.

Case Examples

During the course of this study, two unidentifiable mushrooms involved in poisoning cases were brought to our laboratory for identification. The first case involved a juvenile canine that showed clinical symptoms of mushroom poisoning and what appeared to be a smashed mushroom stipe. The specimen was received from the CAHFS Laboratory at UC Davis. The second case involved multiple pieces of mushroom pileus potentially involved in a human poisoning in Mariposa County, CA. The specimens from both cases were tested on the *Amanita* macroarray, and the ITS regions were sequenced as previously described. The specimen involved in the human poisoning was tested for α-amanitin by the CAHFS Laboratory at UC Davis.

Results

Specimen Identification and Sequencing

Mushroom specimens were initially identified based on morphological characteristics such as attachment of the lamellae, presence and type of veil, spore color, and habitat (22,23). DNA sequences of the ITS region of rDNA were also used as an aid in identification. The ITS1-F and ITS4-B primers produced a 700- to 800-bp-sized amplicon. Sequences were either identified based on their alignment with known species in our own database or compared with sequences in GenBank using the basic local alignment search tool feature (National Center for Biotechnology Information). All specimens used during this study are listed in Table 2.

α-Amanitin Testing

Several mushroom species and two spore prints were tested for α-amanitin by the CAHFS Laboratory at UC Davis using LC/MS. α-Amanitin was detected in specimens of *A. phalloides* and *A. ocreata*. α-Amanitin was not detected in *A. lanei*, *A.* aff. *lanei*, or *A. velosa*. One *A. muscaria* and two unidentified *Amanita* species did not contain detectable α-amanitin. Two spore prints were obtained from *A. phalloides*. One was obtained by placing the mushroom pileus directly on an index card and the other was obtained by suspending the mushroom pileus above an index card so no tissue would contaminate the spores. Both spore prints tested positive for α-amanitin.

Probe Screening and Temperature Optimization

The *Amanita* macroarray was performed at least twice for each species at 50–55°C. Based on the strength of the probe signals at each tested temperature, the optimal hybridization temperature was determined to be 54°C. The three most robust probes for each species were then chosen based on macroarray results at 54°C (Table 3, Fig. 1).

Mixtures of Mushroom Tissue

The mixture of *A. phalloides* and *A. lanei* resulted in binding to all three probes specific for *A. phalloides* (P2, P8, P11) and weak binding to the two probes specific for both *A. lanei* and *A.* aff. *lanei* (L + Laff2C, L + Laff3B). *Amanita phalloides* was detected as expected. However, *A. lanei* did not show binding to the three probes specific to that species (L1, L3, L6). The *A. phalloides* and *A. lanei* mixture also developed weak false-positive binding (cross-hybridization) to one probe specific for *A.* aff. *ocreata* (OO8). The results were consistent for both membranes tested.

The *A. ocreata* and *A. lanei* mixture showed binding to all three probes specific for *A. ocreata* (O8B, O12, O13) on both membranes and weak binding to one probe specific for both *A. lanei* and *A.* aff. *lanei* (L + Laff2C) on one membrane. This mixture also produced weak false-positive binding (cross-hybridization) to probe OO8, which is specific for *A.* aff. *ocreata*, on one membrane. Binding to the probes specific to *A. ocreata* was as expected. Binding to the probes specific for *A. lanei* (L1, L3, L6) was expected but not observed.

The mixture of three species, *A. phalloides*, *A. ocreata*, and *A. lanei*, showed binding to all three probes specific to *A. phalloides* (P2, P9, P11) and all three probes specific to *A. ocreata* (O8B, O12, O13). No binding to any of the probes specific for *A. lanei* was observed, and weak false-positive binding (cross-hybridization) to probe OO8, which is specific for *A.* aff. *ocreata*, was observed.

The final mixture of four mushroom species, *A. phalloides*, *A. ocreata*, *A. lanei*, and *T. magnivelare*, showed binding to all three probes specific for *A. phalloides* (P2, P9, P11) and all three probes specific for *A. ocreata* (O8B, O12, O13). Binding to the probes specific for *A. lanei*, however, was expected but was not

TABLE 2—*Mushroom specimens used in this study, specimen ID, specimen origin, and GenBank accession numbers of the internal transcribed spacer region (ITS) and large subunit (LSU).*

			GenBank Accession No.	
Species	Specimen ID	Origin	ITS	LSU
Amanita franchetii	07-040	Sonoma Co., CA	GQ250398	GQ250413
Amanita gemmata	07-045	Sonoma Co., CA	GQ250399	GQ250414
Amanita lanei	07-036	Sonoma Co., CA	GQ250400	GQ250415
Amanita aff. *lanei*	07-020	Amador Co., CA	GQ250401	GQ250416
Amanita muscaria	07-086	Sonoma Co., CA	GQ250402	GQ250417
Amanita novinupta	07-063	Sonoma Co., CA	GQ250403	GQ250418
Amanita ocreata	07-002	Sonoma Co., CA	GQ250404	GQ250419
Amanita aff. *ocreata*	07-090	Oregon*	GQ250405	GQ250420
Amanita pantherina	09-013	Monterey Co., CA*	GQ401354	GQ401355
Amanita aff. *pantherina*	08-001	Marin Co., CA	GQ250406	GQ250421
Amanita phalloides	07-060	Sacramento Co., CA*	GQ250407	GQ250422
Amanita silvicola	07-061	Sonoma Co., CA	GQ250408	GQ250423
Amanita sp. 1	07-053	Sonoma Co., CA	GQ250410	GQ250425
Amanita sp. 2	07-054	Sonoma Co., CA	GQ250411	GQ250426
Amanita sp. 3	07-075	Sonoma Co., CA	GQ250412	GQ250427
Amanita velosa	07-004	Solano Co., CA	GQ250409	GQ250424

*Specimen sent to laboratory. Exact location of origin unknown.

TABLE 3—*Macroarray oligonucleotide probe sequences, target species and detection, probe melting temperature, and probe-membrane positions on array.*

Position	Oligo Name	Target Species	Sequence (5′-3′)	Tm* (°C)
A1,A3,A5,A7,C1,E1, G1,I1,K1	ITS1-F[†]	Fungi	CTTGGTCATTTAGAGGAAGTAA	50
A2,A4,A6,A8,B1,D1,F1,H1,J1,L1	ITS4-B[†]	Basidiomycetes	CAGGAGACTTGTACACGGTCCAG	59
E6,H8	P2	*Amanita phalloides*	CTTGAGAAGTTGAAAATCTGGGTGTC	56
E7,H6	P 9	*A. phalloides*	GCTGTCTAACTGTGACTGTCTGT	55
E8,G7	P11	*A. phalloides*	ATATGGATGGGGACAACTTGACC	55
D5,I2	O8B	*Amanita ocreata*	GGAGCAATGTCAATTCTCTCTGCTG	58
D6,G5	O12	*A. ocreata*	CTGTACAAAAAGGATGACTTGACCAAC	57
D7,H4	O13	*A. ocreata*	CTCTGCTGTCTAACCTAACAGTTGTC	58
E2,H7	OO3	*A.* aff. *ocreata*	GTTGAAATCTAGGTGTCTATGCC	54
E3,I8	OO7	*A.* aff. *ocreata*	GACCTGTCTGATTATGATAGGTATTGG	57
E4,G2	OO8	*A.* aff. *ocreata*	TGGTGTGATAAAACATATCAATGCC	53
B7,I4	L1	*Amanita lanei*	GCTGTCTAGTAATATGCACGCCT	55
B8,I7	L 3	*A. lanei*	GCATCTTTGTGGCCCATTAATAT	52
C2,I5	L 6	*A. lanei*	AGTCATTGCTGACGGCTGTTGAA	55
C3,G4	[‡]L8 (l + l aff 1B)[§]	*A. lanei* (*A.*aff. *lanei*)	GAGGCTGTTGCTGTCTAGTAATATGC	58
B2,G3	[‡]Laff4[§]	*A. lanei* (others)	TGCGATAAGTAGTGTGAATTGCAG	54
B3,H5	Laff6	*A.* aff. *lanei*	TGGCGGCTGGTTGAAGCTCA	56
B4,I6	Laff7	*A.* aff. *lanei*	CCTTGAGGAACGAAATGTTGGTG	55
B5,H3	Laff8	*A.* aff. *lanei*	GTAGTTCCCCCCCTTTGCTATC	57
C5,G6	L + Laff2C	*A. lanei* & *A.* aff. *lanei*	TGCTGTGTGGGGACTTATTTCTTC	56
C6,I3	L + Laff3B	*A. lanei* & *A.* aff. *lanei*	CTTTGTCTGTCTATGAATCACATAACC	55
C8,G8	V3B	*Amanita velosa*	CTGCCTGTAGACACTCTCTGTGTCTA	60
D2,J2	V6	*A. velosa*	TCTTGCTGGTATGTTTGAGCCAG	55
D3,H2	V7C[§]	*A. velosa* (07075)	CTGACTTGAGTGTCTCGGCTTCTAC	59
B6,C4,C7,D4, D8,E5,F2-8, J3-8,K2-8,L2-8	Buffer	None	N/A	N/A

*Tm = melting temperature of probe (18).
[†]Internal transcribed spacer primer sequences included on macroarray as positive controls (12).
[‡]Two additional probes were included on the final membranes and were later removed from consideration. They are included in this chart for discussion purposes.
[§]Indicates that cross-hybridization (nontarget binding) was seen with the species/specimen listed in the "Target Species" column in parentheses.

observed. The addition of *T. magnivelare* to the mixture did not interfere with the hybridization of the other species.

Sensitivity and Specificity

The *Amanita* DNA array successfully detected purified PCR product from DNA of *A. phalloides* and *A. ocreata* at 0.5 ng/μL and higher. Amplified ITS rDNA from the following mushroom species did not hybridize with the probes designed for *A. lanei, A.* aff. *lanei*, *A. ocreata*, *A.* aff. *ocreata*, *A. phalloides*, or *A. velosa*: *A. franchetii* (07040), *A. gemmata* (07045), *A. muscaria* (07042), *A. novinupta* (07063), *A.* aff. *pantherina* (08001), *A. silvicola* (07061), *A.* sp. (07053), *A.* sp. (07054), *A. xanthodermus* (09001), *C. nuda* (08006), *P. cubensis* (10B), *T. magnivelare* (07064), and *V. speciosa* (08004). Only DNA from *Amanita* sp. (07075) hybridized with *A. velosa* probe V7C (Table 3).

Case Examples

According to results from the developed macroarray, the mushroom specimens involved in the dog and human poisonings were both identified as the toxic *A. ocreata*. Both results were confirmed

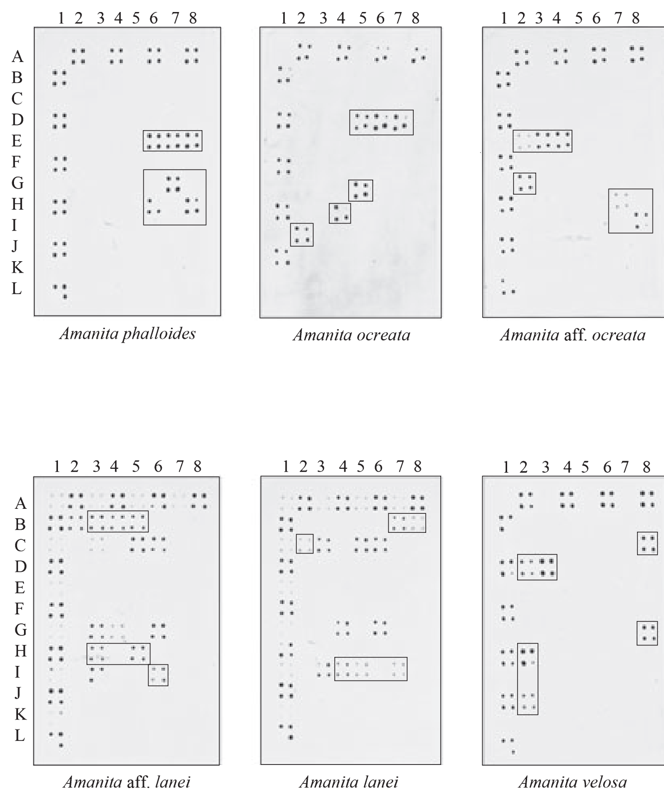

FIG. 1—Amanita *DNA macroarray hybridization patterns for* A. phalloides, A. ocreata, A. *aff.* ocreata, A. *aff.* lanei, A. lanei, *and* A. velosa. *The positive controls are on the top and left side of each membrane.*

through DNA sequencing of the ITS region of rDNA. The specimen involved in the human case tested positive for α-amanitin.

Discussion

The *Amanita* DNA array resulted in fast and accurate identification of the chosen mushroom species. This array was designed to aid in the detection/identification of mushrooms that would otherwise be unidentifiable based on morphological characteristics alone. A macroarray designed for this purpose would be a valuable resource for mushroom identification in medical, veterinary, and mycological diagnostic situations. As expected, the ITS region of rDNA proved to be highly variable for mushrooms within the genus *Amanita*. The array included two toxic mushroom species and three edible species that are commonly encountered in California. The edible species are morphologically similar to the toxic species and are often collected for the table by amateur mushroom hunters. While only five species were targeted for this array, the array could be expanded relatively easily to include any number of species. The addition of other species of *Amanita* and mushrooms belonging to other genera would be greatly beneficial.

The macroarray was designed based on the highly variable ITS region of rDNA amplified using primers ITS1-F and ITS4-B, which are specific to basidiomycetes, a group of fungi that includes mushrooms. Amplification of the unknown DNA using these primers affirms that the sample in question is a basidiomycete. From there, the amplified ITS region of rDNA was hybridized with species-specific probes on a membrane. Complementary binding of the amplified DNA and the probes fixed on the membrane allowed detection and/or identification of the mushroom in question.

Because intraspecific variation in species of *Amanita* may occur, three probes were selected for each chosen mushroom species to eliminate the possibility of a false negative. In addition, the limited number of species of *Amanita* in our collection and the limited number of sequences in GenBank surely do not represent all species of *Amanita* in California. Hence, the specificity of the probes is not absolute. Also, because the probes were designed in part using the somewhat limited sequences deposited in GenBank, it is possible that a new species or a species that has yet to be deposited could hybridize to one or more probes designed in this study. For these reasons, hybridization to all three probes is necessary for a positive identification.

In the analyses with combinations of DNA extracted from multiple mushrooms, DNA from *A. phalloides* and *A. ocreata* was successfully detected. However, *A. lanei* was not consistently detected for unknown reasons during mixture testing even though it was successfully detected using single-species DNA extracts. It is possible that the dried *A. lanei* specimen used for mixture testing was more deteriorated than the specimens of the other two species. Alternatively, perhaps the quality of DNA from the *A. lanei* specimen did not allow robust amplification.

The optimal 54°C hybridization temperature was selected because the temperature was high enough to reduce nonspecific binding and low enough to allow strong visualization of specific binding. However, cross-hybridization was still observed during specificity testing. For example, DNA of mushroom specimen *Amanita* sp. (specimen 07075 from California) hybridized to probe V7C, which was designed to be specific for *A. velosa.* Alignment of the ITS sequence of *Amanita* sp. (07075) and probe V7C showed that the two differed by two base pairs. One mismatch was located close to the end of the probe, while the other was toward the center of the probe. Raising the hybridization temperature might eliminate this and other potentially similar cases of cross-hybridization. However, the strength of a specific binding signal would likely suffer as a result. This highlights the need for more than one probe to constitute an identification and the need to control the specific hybridization conditions of this assay.

Results of the α-amanitin tests for various mushroom species were as expected—α-amanitin was detected in both *A. phalloides* and *A. ocreata*. In addition to testing mushroom specimens, two spore prints from two different specimens of *A. phalloides* tested positive for α-amanitin. To our knowledge, this is the first report of α-amanitin detected in the spores of *A. phalloides* or any other mushroom.

While the macroarray proved to be a successful method of mushroom identification in this study, the idea of designing a real-time PCR assay for the same purpose has been discussed. Basidiomycete-specific primers that produce a shorter amplicon could be used along with species-specific probes in a TaqMan assay. A real-time PCR assay might make a mushroom identification faster and simpler to perform.

Overall, the *Amanita* DNA array proved to be a fast and accurate method of identification for the toxic *A. phalloides* and *A. ocreata* and the morphologically similar edible *A. lanei, A.* aff. *lanei*, and *A. velosa.* This is particularly important for mushroom ingestion cases, whether they involve a person or a pet, because identification might help health care professionals and veterinarians be more prepared for their patient's progress/prognosis.

Acknowledgments

The authors thank Terry Spear and Dr. Robert Rice for advising and comments; Fred Tulleners for advising and

continued support; Dr. Ning Zhang for guidance and assistance in troubleshooting; Celeste Gilbert for instruction; Dr. Matthew Smith for comments and DNA samples from *Amanita* specimens; Karina Perez for continued laboratory support and specimen collection; Dr. Birgit Puschner and Dr. Robert Poppenga from the CAHFS Laboratory for their interest and testing services; Bob Sommer and Michael Sampson for specimen collection; and the Sonoma County Mycological Association.

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Additional information and reprint requests:
R. M. Davis, Ph.D.
Department of Plant Pathology
University of California
Davis, CA 95616
E-mail: rmdavis@ucdavis.edu