



Analytical Methods

DNA barcoding for identification of consumer-relevant mushrooms:
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ABSTRACT

One challenge in the dietary supplement industry is confirmation of species identity for processed raw materials, i.e. those modified by milling, drying, or extraction, which move through a multilevel supply chain before reaching the finished product. This is particularly difficult for samples containing fungal mycelia, where processing removes morphological characteristics, such that they do not present sufficient variation to differentiate species by traditional techniques. To address this issue, we have demonstrated the utility of DNA barcoding to verify the taxonomic identity of fungi found commonly in the food and dietary supplement industry; such data are critical for protecting consumer health, by assuring both safety and quality. By using DNA barcoding of nuclear ribosomal internal transcribed spacer (ITS) of the rRNA gene with fungal specific ITS primers, ITS barcodes were generated for 33 representative fungal samples, all of which could be used by consumers for food and/or dietary supplement purposes. In the majority of cases, we were able to sequence the ITS region from powdered mycelium samples, grocery store mushrooms, and capsules from commercial dietary supplements. After generating ITS barcodes utilizing standard procedures accepted by the Consortium for the Barcode of Life, we tested their utility by performing a BLAST search against authenticate published ITS sequences in GenBank. In some cases, we also downloaded published, homologous sequences of the ITS region of fungi inspected in this study and examined the phylogenetic relationships of barcoded fungal species in light of modern taxonomic and phylogenetic studies. We anticipate that these data will motivate discussions on DNA barcoding based species identification as applied to the verification/certification of mushroom-containing dietary supplements.

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1. Introduction

The dietary supplement industry has grown from \$4 billion in 1994 to an estimated value of \$35 billion in 2015 in the United States ("Nutrition Business Journal," NBJ, 2014; Sarma, Giancaspro, & Venema, 2016), and many "mushroom" containing dietary supplements are formulated with one to several fungal species. The world production of mushrooms for this industry has been estimated to be around \$18 billion, and their trade has been compared to the value of coffee sales worldwide (Chang, 1999; Wasser, Sokolov, Reshetnikov, & Timor-Tismanetsky, 2000). In 2002, the global market value of mushrooms in dietary supplement was approximated to range from \$5–6 billion (Wasser, 2002). The regulation of these products varies depending

on country, but in the United States, they are essentially regulated as a category of foods. The burden of proof of the integrity of these products is borne by the manufacturer. Contamination or adulteration of a product could pose a serious threat to the health of the consumer. Moreover, from a business perspective, such problems could, in turn, have a negative impact on a product line or brand.

One challenge in the dietary supplement industry is the confirmation of the species of the materials. While this challenge has been well documented for herbal (i.e. plant) materials (Cowan, & Fay, 2012; Newmaster, Grguric, Shanmughanandhan, Ramalingam, & Ragupathy, 2013; Stoeckle et al., 2011), it can be particularly problematic for samples that contain fungal mycelia, where morphological characteristics can often be cryptic (Hawksworth, 2004). Moreover, even if morphology could be discerned, for example from mushroom fruiting bodies, the processing of the materials (i.e. drying, milling, etc.) before reaching the vendor essentially eliminates this option. Even organoleptic

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techniques, often used for identifying certain macrofungi (Hanson, 2008), do not work successfully for identification of fungal cultures. Despite those obvious challenges, most regulators worldwide would consider species identity as a critical requirement of good manufacturing practices ("Good Agricultural and Collection Practice for Herbal Raw Materials," American Herbal Pharmacopoeia, 2006). Indeed, it seems that only morphology, often via the supplier/local grower, has been used to provide the scientific identity of fungal products. This could lead to misidentification, and hence, mislabeling of such commodities.

Identification of fungal taxonomy based on morphology is important, but can be misleading due to several factors, such as hybridization (Olson & Stenlid, 2002), cryptic speciation (Harrington & Rizzo, 1999; Kohn, 2005), and convergent evolution (Brun & Silar, 2010). Moreover, it has been common practice in the field of mycology to assign the asexual and sexual stages of the same fungus different Latin binomial names, because they can be completely different morphologically (termed 'pleomorphy') (Sugiyama, 1987); thankfully, this practice is being phased out (Hawksworth, 2012; Hibbett & Taylor, 2013; Taylor, 2011). As a consequence, sequence or DNA-based identification methods have emerged for distinguishing between species among several phyla within the megadiverse fungi (Stajich et al., 2009). Relevant to pleomorphy, the DNA are identical, regardless of sexual or asexual state, further enhancing the utility of DNA based methods for taxonomical identification.

The term "DNA barcoding" was coined originally for species identification in animals (Hebert, Cywinska, Ball, & DeWaard, 2003; Hebert, Ratnasingham, & deWaard, 2003). DNA barcoding systems employ a short standardized region (between 400 and 800 base pairs) to identify species (Kress & Erickson, 2012). The premise of DNA barcoding was that interspecific variation should exceed intraspecific variation. Thus, this difference (i.e. the barcode gap) of a standardized region could be exploited for species level identification (Hebert, Cywinska et al., 2003; Hebert, Ratnasingham et al., 2003). DNA barcoding has been applied to the authentication and identification of plant materials in herbal supplements (Baker, Stevenson, & Little, 2012; Heubl, 2013; Li et al., 2012; Little & Jeanson, 2013; Newmaster et al., 2013; Simmler et al., 2015; Stoeckle et al., 2011; Sucher & Carles, 2008); however, there have been no reported studies on DNA barcoding of fungi in powders sold as dietary supplements, and only a few studies have used DNA barcoding of commercial mushroom products and/or those utilized for their purported medicinal benefits (Dresch et al., 2015; Hapuarachchi, Wen, Deng, & Kang, 2015; Liao et al., 2015; Richter, Wittstein, Kirk, & Stadler, 2014). For example, Atsumi, Kakiuchi, and Mikage (2007) used ITS barcoding to identify sclerotia and cultures of *Wolfiporia cocos*, a fungus used for medicinal properties in Japan. Similarly, these tools have been utilized for the species identification of fruit bodies in Basidiomycetes (i.e. mushrooms) obtained from grocery stores (Dentinger & Suz, 2014) and gourmet mushrooms harvested from the wild (Khaund & Joshi, 2014). Given the numerous manuscripts on DNA barcoding of many different forms of life, the application of these techniques to fungi seems timely, especially toward answering pragmatic questions surrounding product identity and integrity.

From a basic science perspective, the use of molecular data in identification of fungi arose about 20 years ago with the seminal work on nuclear fungal ribosomal operon primers by White, Bruns, Lee, and Taylor (1990). The fungal sequences generated from these primers for the large subunit (nrLSU-26S or 28S), small subunit (nrSSU-18S), and the 5.8S rRNA gene (including the Internal Transcribed Spacer region, ITS1 and ITS2) ushered in a new era of molecular phylogenetic sequence identification in the Kingdom fungi (Bruns, White, & Taylor, 1991; Seifert, Wingfield, & Wingfield,

1995). Recently, a multinational, collaborative consortium of mycologists evaluated six DNA regions for potential fungal barcodes. The mitochondrial gene, which encodes for the cytochrome oxidase 1 (COX1) and is utilized as a barcode marker for the animal kingdom, was deemed inappropriate as a barcoding marker in fungi (Schoch et al., 2012), including mushrooming forming fungi (Dentinger, Maryna, & Moncalvo, 2011). This consortium chose the ITS as a universal barcode for the fungal kingdom (Schoch et al., 2012), which encompassed 17 fungal lineages, including the Ascomycota and Basidiomycota, which are the largest phyla within the kingdom fungi (Hibbett et al., 2007). Cogent to the aims of this study, these two phyla include the greatest number of fungi utilized in dietary supplements (Rogers, 2011; Wasser et al., 2000). Additional studies have provided support for the ITS region as a suitable fungal barcode (Dentinger et al., 2011; Kelly et al., 2011; Seena, Pascoal, Marvanová, & Cássio, 2010). The rise of ITS DNA barcoding approaches to species-level identification of fungi represents a noteworthy advance, which could be of benefit to the dietary supplement industry, particularly with respect to addressing questions of quality, consistency, and integrity of products containing fungal materials.

Dietary supplements that contain fungal products are consumed widely due to their purported antioxidant, anticancer, antimicrobial, and immunomodulating properties (Ganeshpurkar, Rai, & Jain, 2010; Rogers, 2011; Wasser et al., 2000). They play a vital role in the dietary supplement industry and are included in hundreds of diverse products (Ganeshpurkar et al., 2010; Lindequist, 2013; Lindequist, Niedermeyer, & Julich, 2005). Taxonomic identification of these fungal products is therefore a critical need. Accurate species identification can unlock important information regarding a species, and its possible biochemical properties, and thus provide insights into the integrity of samples sold as dietary supplements, further adding to the assurance of quality, value, and safety.

The goal of the present study was to test the utility of fungal ITS DNA barcoding to the identification of mushrooms sold for medicinal value or as commercial edible species to address the following two questions: 1. Is it feasible to extract high quality DNA and sequence the ITS region from processed mushroom powders used in medicinal mushroom dietary supplements? 2. What are the pros and cons of fungal ITS barcoding as it pertains to the fungal based dietary supplements? To address these questions, fungal samples were analyzed from: 1) vendors that grow and supply medicinal mushrooms (as powdered mycelium) for the manufacturing of dietary supplements; 2) commercially available mushroom dietary supplements (from capsules) representing two different companies; and 3) grocery store mushrooms. If these techniques could address such questions, then it was the goal to apply them in the authentication (i.e. verification of species identities) of fungal materials used in commercial products.

2. Materials and methods

2.1. Sampling

Three different types of mushroom samples were analyzed (Fig. 1), including 20 powdered mushroom mycelium samples obtained by New Chapter Inc. from a commercial supplier that were under consideration for future use; six edible mushrooms sold in grocery stores; and seven samples of fungal powder obtained from capsules from two different companies that sell mushroom dietary supplement finished products. All of these were analyzed via fungal DNA barcoding (Tables 1 and S1).

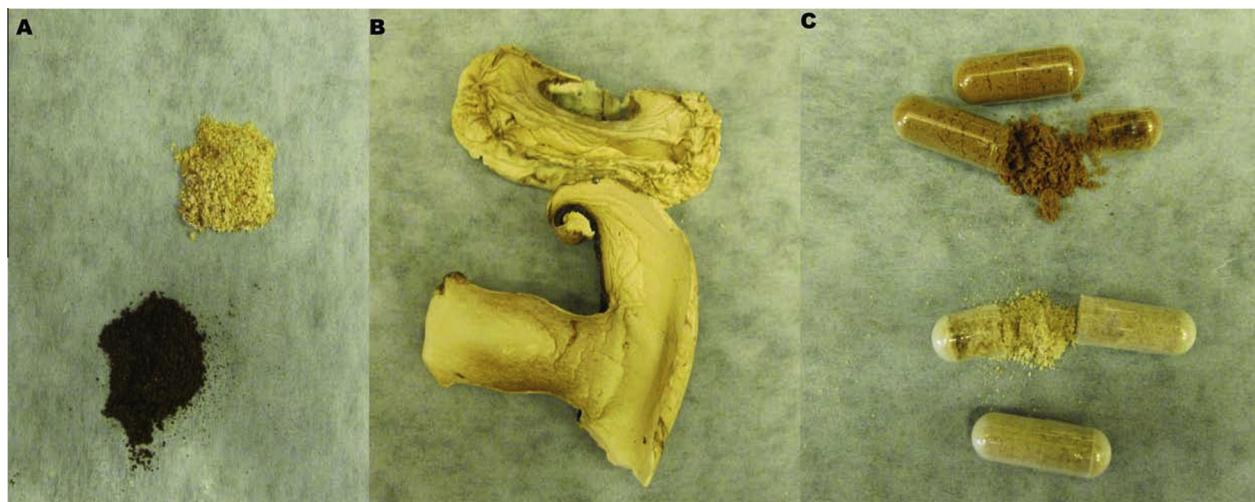


Fig. 1. Different mushroom sample types from which ITS sequences were obtained. 1A: Mushroom mycelium powders that were both labeled *Lentinula edodes* obtained from a vendor of powdered mycelia. DNA barcoding verified the identity of the top sample; however, we were unable to obtain quality DNA from the bottom sample, presumably due to denaturing from heat treatment. 1B: An example of a 'mushroom' purchased from a grocery store; DNA barcoding of a small piece of the fruiting body identified this as *Agaricus bisporus*. 1C: Dietary supplement capsules that were labeled to contain *Ganoderma lucidum* (top), and *Cordyceps sinensis* (bottom). Here, the different colors indicate powders that contained different fungal species. ITS barcoding identified these as *Ganoderma sichuanense* ('Asian variety of *Ganoderma lucidum*') and *Tolypocladium inflatum*, respectively.

2.2. DNA extractions

For each sample, DNA extraction was performed in duplicate ($n = 2$). The fungal material from all three-sample types (powdered mycelia, grocery store mushrooms, and powder from capsules; Fig. 1) was transferred to a lysis bead tube with DNA lysis buffer (Zymo Research fungal/bacterial DNA extraction kit). Subsequently DNA was extracted using procedures outlined in the Zymo fungal/bacterial DNA Mini Prep. In the final step, 30 μ L of elution buffer was added to the column matrix for elution of DNA.

2.2.1. Powdered mycelia

Two scintillation vials were made from each product number. The vials were filled with mushroom powder from the top and middle layers of the batch. The scintillation vials were labeled with Product name, Lot number, and Batch number, and then stored at ambient temperature. For DNA extractions, approximately 5 mg of mushroom powder were drawn from each of the two scintillation vials.

2.2.2. Grocery store mushrooms

For DNA extractions from mushrooms, a small piece of dried cap (pilus) was removed from the packet and ground to a fine powder with a mortar and pestle using liquid nitrogen. Subsequently, approximately 5 mg of the ground powder was used for DNA extractions.

2.2.3. Capsules from commercial dietary supplements

Mushroom powder was obtained by pulling apart capsules that were contained in each bottle. Ten capsules were emptied into a scintillation vial, and two scintillation vials were prepared (i.e. 10 capsules/scintillation vial; $n = 2$ for each commercial dietary supplement). Each scintillation vial was labeled with Product name and Lot number and stored at ambient temperature in the laboratory. For DNA extractions, approximately 5 mg of capsule powder were drawn from the scintillation vials.

2.3. PCR amplification and sequencing

The entire ITS region was PCR-amplified on an Applied Biosystems Veriti thermal cycler using PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire, UK) with primers ITS5 and ITS4 (Gardes, White, Fortin, Bruns, & Taylor, 1991; White et al., 1990). The PCR reaction was carried out in 25 μ L containing 3–5 μ L template DNA, 2.5 μ L BSA (New England BioLabs Inc, Ipswich, USA), 2.5 μ L 50% DMSO (Sigma, St Louis, MO, USA), and 1 μ L of each 10 μ M forward (ITS5) and reverse (ITS4) primer (Promputtha & Miller, 2010). The rest of the volume was made up to 25 μ L by adding molecular biology grade H₂O (Fisher Scientific, Waltham, MA, USA). The following thermocycling parameters were used for the amplification: initial denaturation at 95 °C for 5 min followed by 39 cycles of 95 °C for 30 s, 50–52 °C for 15 s, and 72 °C for 1 min with a final extension step of 72 °C for 10 min (Schoch et al., 2012). When PCR amplifications using the above primers and annealing temperatures was not successful, ITS1F/ITS1 primers (Gardes & Bruns, 1993) were used. Negative controls were included to ensure that PCR amplicons were not contaminated. The PCR products were then run on an ethidium bromide-stained 1% agarose gel (Fisher Scientific, Waltham, MA, USA) along with a 1 kb DNA ladder (Promega, Durham, NC, USA) to estimate the size of the amplified band. Prior to Sanger sequencing, PCR products were purified using a Wizard SV Gel and PCR Clean-up System (Promega, Durham, NC, USA).

2.4. Sanger sequencing

Sanger sequencing of the purified PCR products was performed at Eurofins Genomics (<http://www.operon.com/default.aspx>) using BigDye Terminator v3.1 cycle sequencing. The sequences were obtained bidirectionally using both strands with a combination of the following primers: ITS1F (Gardes & Bruns, 1993)/ITS1/ITS5 and ITS4. Sequences were generated on an Applied Biosystems 3730XL high-throughput capillary sequencer. For both sequencing reactions, approximately 15 μ L of PCR template was used along with 2 μ M sequencing primers. Sequences were assembled with Sequencher 5.2.3 (Gene Codes, Ann Arbor, MI, USA), optimized, and then corrected manually when necessary; the latter step was

to insure that the computer algorithm was assigning proper base calls.

2.5. BLAST search

Each sequence fragment was subjected to an individual Basic Local Alignment Search Tool (BLAST) (Altschul, Gish, Miller, Myers, & Lipman, 1990) search to verify identity. BLASTn search was employed using nucleotide collection (nr/nt) with uncultured/environmental samples sequences excluded. Experts in the fungal research community have recently proposed a set of working rules for handling ITS data, which were consulted for accurate identification of fungi using molecular sequence data (Nilsson et al., 2012; Schoch et al., 2014). Briefly, these include: 1) check that all query sequences were representative of the ITS region; 2) check for orientation of the query sequence by performing an alignment with other ITS sequences; 3) check for chimeric sequences via BLASTn search; 4) check for broken sequences via BLASTn search; and 5) verify taxonomic annotations carefully, by using only authentic, published sequences (Nilsson et al., 2012).

The complete ITS sequences were subjected to a BLAST search in NCBI-GenBank (Altschul et al., 1990). Due to lack of an arbitrary percentage sequence similarity that could precisely designate conspecific taxa across the kingdom fungi (Bruns, Arnold, & Hughes, 2008), no one cutoff value has been used for species identification. In the past, Mycologists have used a cutoff value ranging from <3 to 5% for ITS sequence divergence as conspecificity among fungi (Izzo, Agbowo, & Bruns, 2005; Morris, Smith, Rizzo, Rejmanek, & Bledsoe, 2008; O'Brien, Parrent, Jackson, Moncalvo & Vilgalys, 2005; Ryberg et al., 2008; Smith, Douhan, & Rizzo, 2007). For this study, we used query coverage of ≥80% and ≥97–100% sequence similarity (3% sequence divergence) for assigning a species name based on consideration of results from the GenBank BLAST search.

For mushroom fungi, the average intraspecific ITS variability is approximately 3% (Hughes, Petersen, & Lickey, 2009; Nilsson, Kristiansson, Ryberg, Hallenberg, & Larsson, 2008) with a standard deviation of 5.62%; however, this value requires further evaluation, as no single value appears to fit well with morphological based identification for all fungal species (Nilsson et al., 2008). It has been advised that BLAST search identifications against GenBank should be made with caution, as more than 27% of GenBank fungal ITS sequences are insufficiently identified (Nilsson, Ryberg, Kristiansson, Abarenkov, & Larsson, 2006) and about 20% of all fungal sequence in the GenBank are incorrectly annotated (Bridge, Roberts, Spooner, & Panchal, 2003). Therefore, in addition to GenBank, the ITS region was subjected to an additional BLAST search against the curated database termed UNITE (<http://unite.ut.ee/>), which provides identification of ITS sequence as a species hypothesis (Koljalg et al., 2013) based on sequence similarity.

2.6. Phylogenetic analyses

In cases where there was a recent phylogenetic evaluation of the fungal sample analyzed in this study, the newly obtained ITS sequences from fungal samples were aligned with authenticated published sequences that originated from type material (Schoch et al., 2014) or from vouchered herbarium samples (Brock, Döring, & Bidartondo, 2008; Osmundson et al., 2013) using the multiple sequence alignment program, MUSCLE (Edgar, 2004), with default parameters in operation. MUSCLE was implemented using the program Seaview v. 4.1. (Galtier, Gouy, & Gautier, 1996; Gouy, Guindon, & Gascuel, 2010). Maximum Likelihood (ML) analyses were conducted using RAxML v. 7.0.4 (Stamatakis, Hoover, & Rougemont, 2008) if more than 50 sequences were included; analyses were run on the CIPRES Portal v. 3.3 (Miller, Pfeiffer, & Schwartz, 2010) with the default rapid hill-climbing

Table 1

List of fungi sampled in this study for ITS barcoding.

Fungus Name on Label	Common Name	ITS Barcoding ID	Sample codes	GenBank Accessions	GenBank Query Coverage	GenBank Percent Similarity	Color Codes*
<i>Tremetes versicolor</i> (<i>Coriolus versicolor</i>)	Turkey tail	<i>Tremetes versicolor</i> (<i>Coriolus versicolor</i>)	Batch 1 -13316010	KT693226, KT693227	100%	100%	Green
<i>Hericium erinaceus</i>	Lions mane	<i>Hericium erinaceus</i>	Batch 1- 13319025	KT693228, KT693229	97%	99%	Green
<i>Hericium erinaceus</i>	Lions mane	<i>Hericium erinaceus</i>	Batch 2- 13319025	KT693230, KT693231	97%	99%	Green
<i>Inonotus obliquus</i>	Chaga	No sequence obtained	Batch 1- 12272311	ó	N/A	N/A	Gray
<i>Inonotus obliquus</i>	Chaga	<i>Inonotus obliquus</i>	Batch 2 - 13064168	KT693232, KT693233	100%	99%	Green
<i>Grifola frondosa</i>	Maitake	<i>Grifola frondosa</i>	Batch 1- 13059120	KT693234, KT693235	98%	99%	Green
<i>Lentinula edodes</i>	Shiitake	No sequence obtained	Batch 1- 13025126	ó	N/A	N/A	Gray
<i>Lentinula edodes</i>	Shiitake	<i>Lentinula edodes</i>	Batch 2- 14065,14015	KT693236, KT693237	100%	99%	Green
<i>Wolfiporia cocos</i> (<i>Poria cocos</i>)**	Poria	No sequence obtained	Batch 1 - 12294068	ó	N/A	N/A	Gray
<i>Wolfiporia cocos</i> (<i>Poria cocos</i>)**	Poria	No sequence obtained	Batch 2 - 12294068	ó	N/A	N/A	Gray
<i>Wolfiporia cocos</i> (<i>Poria cocos</i>)	Poria	<i>Wolfiporia cocos</i> (<i>Poria cocos</i>)	Batch 3- 14064, 14098	KT693238, KT693239	≥92%	93-95%	Green
<i>Agaricus bisporus</i>	Crimini	<i>Agaricus bisporus</i>	Grocery store - 7941	KT693240, KT693241	98%	99%	Green

<i>Lentinula edodes</i>	Shiitake	<i>Lentinula edodes</i>	Grocery store - 007039	KT693242, KT693243	98%	99-100%	Green
<i>Phellinus linteus</i>	Mesima	<i>Inonotus sanghuang</i>	Batch 1- 12192170	KT693244, KT693245	98%	99%	Yellow
<i>Phellinus linteus</i>	Mesima	<i>Inonotus sanghuang</i>	Batch 2 - 12192170	KT693246, KT693247	98%	99%	Yellow
<i>Ganoderma lucidum</i>	Reishi	<i>Ganoderma sichuanense</i>	Batch 1-13022086	KT693248, KT693249	89%	99%	Yellow
<i>Ganoderma lucidum</i> **	Reishi	No sequence obtained	Batch 2- 12361220	ó	N/A	N/A	Gray
<i>Ganoderma lucidum</i>	Reishi	<i>Ganoderma sichuanense</i>	Batch 3- 14034, 14113	KT693250, KT693251	89%	99%	Yellow
<i>Ganoderma lucidum</i>	Reishi	<i>Ganoderma sichuanense</i>	Batch 4- 13106, 13304, 13309	KT693252, KT693253	89%	99%	Yellow
<i>Boletus edulis</i>	Porcini	<i>Boletus shiyoung</i>	Grocery store - 11407	KT693260, KT693261	≥80%	98-100%	Yellow
<i>Craterellus comucopoides</i>	Black trumpet	<i>Craterellus comucopoides</i>	Grocery store - 11399	KT693262, KT693263	≥80%	98-100%	Green
<i>Cordyceps sinensis</i>	Cordyceps	<i>Tolypocladium inflatum</i>	Batch 1 - 13015080	KT693266, KT693267	98%	99%	Aqua
<i>Inonotus obliquus</i>	Chaga (Sclerotia)	<i>Cladosporium</i> sp.	Batch 1- 13055002	KT693272, KT693273	≥98%	99%	Aqua
<i>Cantharellus cibarius</i>	Chanterelle	No sequence obtained	Grocery store ó Batch1 001063	ó	N/A	N/A	Gray
<i>Cantharellus cibarius</i>	Chanterelle	<i>Cantharellus</i> sp.	Grocery store ó Batch2 001234	KT693264, KT693265	≥98%	99%	Yellow
<i>Cordyceps sinensis</i>	Cordyceps	<i>Tolypocladium inflatum</i>	Batch 1- 092513	KT693268, KT693269	98%	99%	Aqua
<i>Ganoderma lucidum</i>	Reishi	<i>Ganoderma resinaceum</i>	Batch 1- 0121714	KT693256, KT693257	≥98%	≥98%	Yellow
<i>Cordyceps sinensis</i>	Cordyceps	<i>Tolypocladium inflatum</i>	Batch 2- 080213	KT693270, KT693271	98%	99%	Aqua
<i>Ganoderma lucidum</i>	Reishi	<i>Ganoderma resinaceum</i>	Batch 2- 071213	KT693258, KT693259	≥98%	≥98%	Yellow
<i>Cordyceps sinensis</i>	Cordyceps	No sequence obtained	Batch 1- 1410004	ó	N/A	N/A	Gray
<i>Ganoderma lucidum</i>	Reishi	<i>Ganoderma sichuanense</i>	Batch 1- 1406706	KT693254, KT693255	81%	99%	Yellow
<i>Cordyceps sinensis</i>	Cordyceps	No sequence obtained	Batch 2- 1322705	ó	N/A	N/A	Gray
<i>Cordyceps sinensis</i> **	Cordyceps	<i>Inonotus sanghuang</i>	Batch 2 -10221252	KT693274, KT693275	98%	99%	Aqua

Weighted intraspecific ITS variability in the kingdom Fungi was calculated to be 2.51% with a standard deviation of 4.57; while the weighted intraspecific ITS variability for Ascomycota was calculated as 1.96% with a standard deviation of 3.73; and weighted intraspecific ITS variability for Basidiomycota was calculated as 3.33% with a standard deviation of 5.62 (Nilsson et al., 2008).

*Color codes: Green: Binomial name accurate and matches the product label; Yellow: Name accurate at genus level, but taxonomic changes abound at species level; Aqua: Binomial name is incorrectly applied and does not match name on product label; Gray: No sequence was obtained.

**Only samples with accurate identification and support for Latin binomial names (genus and species) were considered for further product development and commercial production.

algorithm and GTR model employing 1000 fast bootstrap searches. Clades with bootstrap values $\geq 70\%$ were considered significant and strongly supported (Hillis & Bull, 1993).

3. Results

A total of 33 samples were analyzed via ITS barcoding (Table 1). A goal was to show the utility of these techniques for scenarios that arise in the dietary supplement industry, especially for a company that wanted to insure the content of its products. This included 20 “vendor samples”, which were essentially mycelium that had been grown on rice, and those materials were subsequently powdered and dried. That scenario was akin to starting materials that could be delivered for processing. Such samples lack morphological characteristics that could be used to identify them. Indeed, they essentially look like a brown powder (Fig. 1A). Additionally, six

samples were purchased from a local grocery store. Whether they were dried or fresh, these samples typically had morphological features that an average consumer would associate with a “mushroom” used in culinary applications (Fig. 1B). Finally, seven samples were purchased from two different vendors of dietary supplements. Essentially, these were capsules that were purported to contain powdered mushroom materials and were finished products available for sale to the consumer (Fig. 1C). In total, these materials provided a breadth of samples to evaluate ITS barcoding, from those that still possessed some discernable morphologically traits to those that were nothing more than powder.

There was some disparity between the samples (Fig. 2). Of all the samples that were analyzed by BLAST search and/or a combination of BLAST and phylogenetic analyses, 30% (10/33) accurately displayed a Latin binomial name on the product label. Alternatively, 30% (10/33) displayed a correct genus name, but the taxonomic name at the species level was incorrect. This

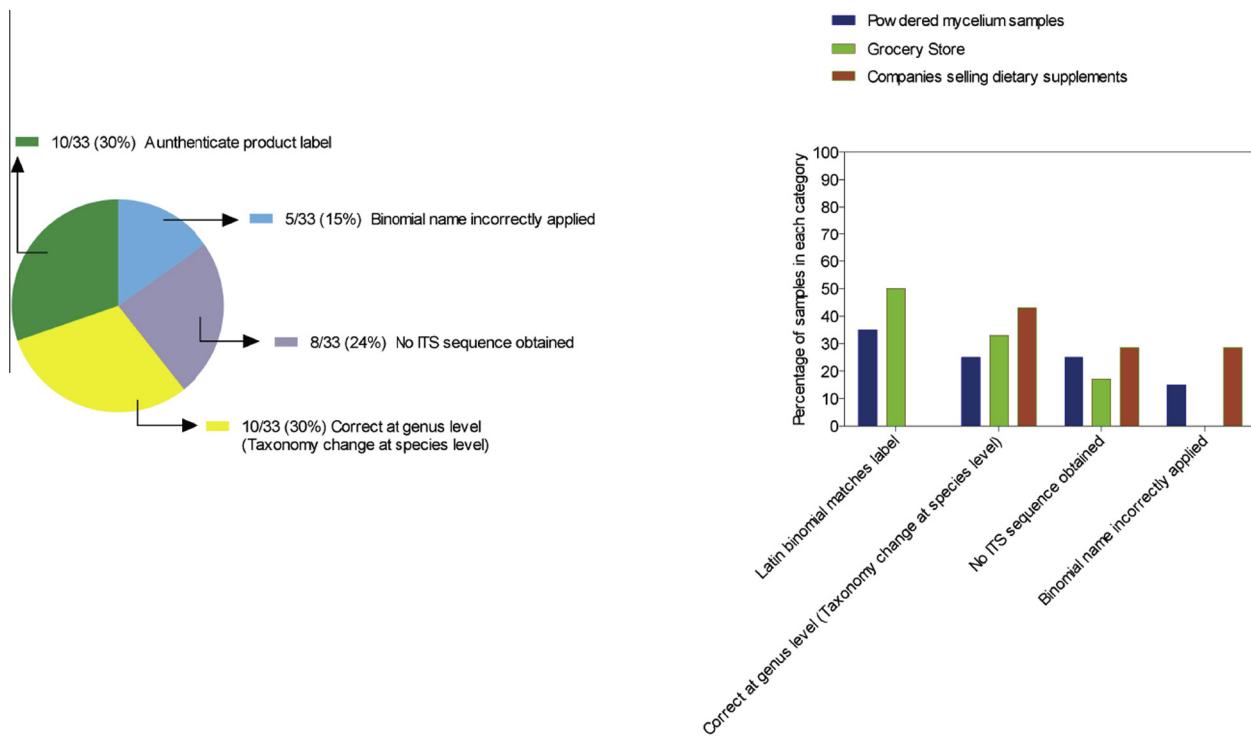


Fig. 2. (left) Species identifications based on DNA barcoding from all samples analyzed ($n = 33$). Color codes match those used in Table 1. (right) DNA barcoding results from 20 powdered mycelium samples, 6 grocery store mushroom samples, and 7 samples from companies that sell mushroom dietary supplements ($n = 33$).

discrepancy could be due to recent refinements in taxonomy in the mycological literature based on molecular methods. Moreover, 15% (5/33) of samples displayed a completely incorrect Latin binomial name on the product label; several of these were quite egregious errors. Interestingly, for 24% (8/33) of samples, ITS sequence data were not obtained, and reasons for this problem were explored, as discussed below.

One of the goals of this study was to evaluate the content of labeled materials, as a vendor qualification program, checking if the label claims matched ITS barcoding. Once conducted, those results were compared to labeled grocery store mushrooms, since they were already in commerce and their morphological traits were more discernable. In those two cases, the Latin binomial on the label matched the ITS barcoding in 35% (7/20) of the powdered mycelia samples and 50% (3/6) of the grocery store mushrooms, where as none of the commercial dietary supplements matched the Latin name on their label. For those that were not accurate to the species level, 25% (5/20) of the powdered mycelia, 33% (2/6) of the grocery store mushrooms, and 43% (3/7) of the dietary supplement capsules were correct at the genus level, but species epithets were inconsistent due to recent taxonomic changes in the mycology literature. However, 15% (3/20) of the powdered mycelia samples and 28% (2/7) of the dietary supplements were incorrectly labeled at both the genus and species levels. With respect to grocery store mushrooms, all of them were accurate at least at the genus level, with several being correct at both the genus and species level (50%; 3/6). Moreover, on average across all three types, it was not possible to acquire quality genomic DNA for about 24% (8/33) (Fig. 2) of the samples, thereby precluding the ability to perform ITS sequencing.

3.1. BLAST search

For samples for which we were able to obtain ITS sequence data, the closest hits of the BLASTn searches are provided along with

notes on species identification and recent taxonomic and nomenclatural changes (Supporting information). Where possible, we have included information of closest hits based on ITS sequence data obtained from either type specimen and/or authenticate vouchered herbarium specimens. The ITS sequences were also aligned and analyzed with highly similar sequences from GenBank BLAST search to count for variable bases in sequenced ITS amplicons. The ITS region was found to show 0.7–4.8% intraspecific variability between the analyzed species (Table S1; Supporting information). All ITS sequences generated from this study were deposited in the GenBank and provided in Table 1 (KT693226–KT693275).

4. Discussion

Fungi represent the second largest kingdom of eukaryotic life on earth. Members of the fungal kingdom play significant roles in human life and have the ability to occupy multiple natural and artificial niches. Identification of fungi to species is paramount in both basic (i.e. ecology, taxonomy) and applied (i.e. dietary supplement industry, genomics, bio prospecting) research areas. This is especially true for the dietary supplement industry, where fungi (typically macrofungi belonging to Ascomycota and Basidiomycota) are utilized routinely for consumption due to their purported medicinal properties (Wasser, 2002). Traditionally, taxonomic mycologists have used morphology (phenotypic characters), such as spore producing structures formed as a result of asexual (mitosis) or sexual (meiosis) reproduction, as a sole means for identifying fungal species (Hyde, Abd-Elsalam, & Cai, 2010), and it is still very much used as a means of species identification within the mycological community. Use of morphology in fungal species identification is important to understand the evolution of morphological characters. However, identifying fungi based on morphology alone can be problematic, especially when non-experts are dealing with ground fungal mycelia powder obtained from the culturing of medicinal mushrooms. Moreover, even for experts, cryptic

speciation is now considered prevalent in fungi, further compounding the challenges with morphological characteristics. Therefore, our goal was to test the utility of fungal barcoding methodology for the identification of three different types of fungal samples at the genus and species level (Fig. 1).

4.1. Challenges of ITS barcoding with powdered mycelia

Most of these challenges arose from powdered mycelia, akin to the types of material a dietary supplement processor may receive from a vendor that grows fungal cultures (Fig. 1A; Table 2). Previous studies on DNA barcoding of powdered herbal/plant products (Baker et al., 2012; Little & Jeanson, 2013; Newmaster et al., 2013) have also encountered similar problems. We postulate that the application of excessive heat to these samples, likely during the drying/milling/manufacturing processes, could be a key factor compounding our ability to acquire genomic DNA. For example, for two samples that were ascribed the same product label from the same vendor, it was not possible to obtain DNA from the one that was darker in color, presumably because it was heated to a greater extent (Fig. 1A, bottom), thereby denaturing the DNA. In addition, due to the fragmented nature of the DNA, we were unable to obtain PCR amplification, as the primer binding sites may have degraded (Little & Jeanson, 2013; Newmaster et al., 2013). While we are not certain of the extent of heating of those samples, these challenges were not observed with the lighter colored sample (Fig. 1A, top).

4.2. ITS barcoding from edible mushrooms from a grocery store

While the primary goal was to test ITS sequencing on powdered mycelium, which completely lack morphological characteristics, a series of six culinary mushrooms were examined in parallel (Table 1) to see how the techniques would work in samples that had fruiting bodies (pileus) intact and evaluate accuracy of their product labels. We were able to PCR amplify and sequence the ITS region from almost all the samples, with the exception of one labeled *Cantarellus cibarius* (Grocery store Batch 1-001063). The PCR amplification of the ITS region of this sample showed two

bands on the agarose gel (Fig. S5a, Supporting information). Sanger sequencing was therefore not obtained for these samples, as it would have resulted in mixed signals, and this precluded the acquisition a good ITS sequence from this sample. The DNA extraction and PCR amplification were repeated, yielding the same results (Fig. S5b, Supporting information); however, we were able to obtain good sequence data from another batch of the same mushroom from the same grocery store purchased at a later date (Grocery store Batch 2-001234). Reasons for this disparity are unknown. For 50% of the samples (3/6), it was possible to use ITS data to unambiguously assign Latin binomial names (Fig. 2). Interestingly, one species that was ascribed *Boletus edulis* on the product label was identified as a recently described species, *Boletus shiyong* (Dentinger & Suz, 2014) based on both ITS BLAST search as well as phylogenetic analyses (Table 1; Fig. S3, Supporting information). Dentinger and Suz (2014), sequenced the ITS region from "Porcini" grocery store mushrooms from London (U.K) and identified three new species. Our ITS barcoding efforts demonstrate that one of the three species they noted (i.e. *B. shiyong*) was also present in a US grocery store, but sold under the label *B. edulis*. Taken together, these data demonstrate the applicability of fungal ITS barcoding not only for challenging powdered mycelia samples, but also for more commonly observed 'mushrooms' in the food chain, as suggested previously by Dentinger and Suz (2014).

4.3. ITS barcoding from commercial fungal dietary supplement capsules

After observing the challenges in acquiring quality genomic DNA from processed powdered mycelium samples, we sought to examine how far in the processing chain these barcoding techniques could be applied by examining seven purchased samples of commercial dietary supplements that were labeled to contain fungal materials and sold in capsule form (Table 1). These included two different companies that sold products labeled to contain *Ganoderma lucidum* and *Ophiocordyceps sinensis* (marked on label as *Cordyceps sinensis*). It was possible to sequence the fungal DNA in these samples; however, the results from ITS barcoding showed that *Ganoderma lucidum* was not present in the dietary supplement

Table 2

Outline of potential problems, consequences and solutions for ITS barcoding fungal samples for use in dietary supplements and/or culinary mushrooms.

Problem	Consequence	Solution/recommendation
Mixed mushroom sample in powder	DNA extraction works, but PCR amplification will fail and multiple PCR bands might be visible. Sanger sequencing will fail	The powder should contain only one mushroom sample. Mixed samples will not work for ITS barcoding with Sanger sequencing. For mixed samples, which could be due to contamination, substitution, and fillers (Newmaster et al., 2013); identification could be made via Next Generation Sequencing Technologies (Ivanova, Kuzmina, Braukmann, Borisenko, & Zakharov, 2016)
Samples too baked or processed with high heat	The samples cannot be baked for a long time via heat treatment. Too much dry heat can breakdown DNA. As DNA becomes shredded into short fragments, ITS barcoding is challenging, as PCR amplification does not work efficiently because primer-binding sites are likely degraded	The samples should undergo minimal processing. This ensures the DNA is not fragmented and PCR amplification is effective. ITS barcoding technique should be utilized on starting material to ensure quality control. The barcoding technique is more powerful for testing name identifications when used on starting material, as extraction of DNA from finished product may have deteriorated and damaged the DNA. ITS barcoding should not be tested on finished products, as sample-processing treatment may affect quality and quantity of DNA. When possible users should refer to ITS RefSeq database (Schöch et al., 2014) in GenBank, which consists of fully annotated ITS sequences obtained by expert mycologists from holotype and ex-holotype cultures. The sequences are authenticated and are published in peer-reviewed Mycology journals. However, for edible mushrooms and fungi used in dietary supplements, the RefSeq database is not as powerful, since these fungi are not deposited in this database. Therefore, in this study, we used the UNITE database, which is curated and contains annotated ITS sequences based on species hypothesis (Koljalg et al., 2013)
Reference Database for BLAST search	Identification should be made with caution, as more than 27% of GenBank ITS sequences are insufficiently identified (Nilsson et al., 2006) and about 20% of all fungal sequence in the GenBank are incorrectly annotated (Bridge et al., 2003). Perhaps most importantly, only 21% of ITS sequences available in the GenBank are tied to a vouchered herbarium specimen (Ryberg et al., 2009). Using erroneous ITS data in GenBank may lead to inaccurate conclusions of sequenced fungal samples	When using the nucleotide (nr/nt) database in GenBank, the users should only refer to published sequences from Mycological studies

capsules (Table 1). Instead, the capsules consisted of two other species of *Ganoderma*, namely: *G. resinaceum* and *G. sichuanense* (often termed the 'Asian variety of *G. lucidum*' (Richter et al., 2014)) based on the BLAST search results via GenBank and ML phylogenetic analysis (Fig. S2, Supporting information). Moreover, a *Cordyceps sinensis* labeled product was shown to contain *Tolypocladium inflatum*, which is a species that is in the same order, Hypocreales, as *C. sinensis*, but a completely different genus (Fig. S4, Supporting information). Also, it was not possible to obtain DNA from two of the commercial capsule samples (Batch 1 – 1410004, and Batch 2 – 1322705), likely due to the following reasons: 1) DNA from highly processed finished material can often be damaged into short fragments leading to poor and fragmented DNA sizes, which cannot be successfully utilized for downstream applications such as PCR and Sanger sequencing; and 2) the product may contain DNA from mixed samples thus hindering PCR amplification (Newmaster et al., 2013). Some possible solutions to these problems are outlined in Table 2. For the two samples labeled *Cordyceps sinensis* where we could not obtain DNA, we were unable to distinguish if the capsule powder was highly processed or contained mixed material in the form of contaminants. Thus, when considering the use of ITS sequencing for dietary supplement purposes, it is likely prudent to obtain DNA from samples prior to sample processing to avoid any misconceptions about ITS fungal barcoding of finished or processed dietary supplements sold as capsules.

4.4. Pros and Cons of ITS region for fungal barcoding

The benefits of the ITS region are that it is multiple copy (~250 copies) in the genome (Vilgalys & Gonzalez, 1990); therefore it allows for easy amplification and sequencing in most fungal species, particularly those of the Dikarya (i.e. Ascomycota and Basidiomycota), which include the majority of fungi used in both edible and dietary supplement products. A large (300,000 ITS sequences) reference database exists in GenBank (Koljalg et al., 2013) representing approximately 14,000 species (Schoch & Seifert, 2011), and any user can submit a query sequence for identification via a BLAST search. Therefore, based on the results of this study and previous studies that have worked on ITS barcoding of mushrooms (Dentinger, Margaritescu, & Moncalvo, 2010; Dentinger & Suz, 2014; Dentinger et al., 2011; Khaund & Joshi, 2014; Osmundson et al., 2013; Schoch et al., 2014), we are of the opinion that the dietary supplement industry would benefit from using the ITS region as a barcoding marker for testing the authenticity of products that include fungi. Moreover, if such a product has plant materials mixed into it, one could still extract and amplify the ITS sequences of the fungal material, since plant-based DNA do not respond to the same fungal specific primers. Indeed, in a recent high profile study where DNA barcoding was used to examine herbal supplements (Smith, 2015), the DNA of rice, often used as a filler, was a noted contaminant (Newmaster et al., 2013), and that should not represent a problem if one were seeking the fungal ITS sequence.

Although the ITS region performs well as a suitable fungal barcoding marker, it is met with criticism (Kiss, 2012). The ITS region does not work well in some highly speciose genera, such as *Penicillium*, *Aspergillus*, *Cladosporium*, and *Fusarium* as these taxa have narrow barcode gaps in their ITS regions (Al-Hatmi et al., 2016; Samson & Pitt, 2000; Samson et al., 2014; Schoch et al., 2012; Stielow et al., 2015). Intrageneric ITS variation does occur in some mushroom fungi (Basidiomycota) (Chen et al., 2016; Lindner & Banik, 2011). Thus, it is likely beneficial to carry out such studies while working with scientists familiar with the ever changing mycological literature and standards. For example, about 20% of all fungal sequence in the GenBank are incorrectly annotated (Bridge et al., 2003). Perhaps most importantly, only 21% of ITS

sequences available in the GenBank are tied to a voucherized herbarium specimen (Ryberg, Kristiansson, Sjokvist, & Nilsson, 2009). Identification using GenBank will not return positive results if the public database is not populated with good quality reference sequences. Hence, that is why this study relied heavily on sequences associated with voucherized materials and/or studies, where possible, that were published in high impact mycological journals.

To minimize the influx of incorrectly identified taxonomic names associated with ITS sequences in GenBank, a collaborative study focused on sequences from the ITS region that were derived from type specimens and/or ex-type cultures (Schoch et al., 2014). The authors re-annotated and verified sequences in a curated public database at the National Center for Biotechnology Information (NCBI), and named it the RefSeq Targeted Loci (RTL) database. A number of medicinal mushrooms, such as those ITS barcoded in this study (Table 1), belong to fungal taxa that do not have ITS barcodes currently in GenBank that are sequenced from their holotype material. In the future the ITS region from these samples could be sequenced and made available in the GenBank by sequencing the type material deposited in the herbarium (Brock et al., 2008). Different research groups that work on DNA barcoding with diverse and economically important groups of fungi have begun to develop ITS databases for fungi, such as those that are human and animal pathogenic fungi (Irinyi, Lackner, De Hoog, & Meyer, 2015) and plant pathogenic fungi (Nilsson et al., 2014), to name a few. It is anticipated that this trend will continue in the future, and several different groups will likely host ITS sequences of fungi prevalent in their respective fields for rapid identification via barcoding. A recently published review on Fungal DNA barcoding lists numerous online database that have been established for generating pairwise alignments for ITS barcoding, in addition to polyphasic identifications using other gene markers (Robert et al., 2015). In this regard, it could be a community interest to assemble a reference fungal ITS database comprised of authenticated medicinal mushroom species. Such a database could be useful in testing the accuracy of species identities of fungal samples used in the manufacturing of dietary supplements.

4.5. Barcoding for sample verification – setting industry standards

The plant/herbal/botanical community has embraced DNA barcoding as a means of authenticating specimens and checking for misidentified names on herbal product labels and have called for establishing an online sequence databases for DNA barcoding of medicinal herbs (Chen et al., 2014). The purpose of this study, in part, was to motivate a discussion on fungal DNA barcoding based species identification, particularly as applied to the verification/certification of mushrooms found in many dietary supplement products. Fungal barcoding techniques can be applied to processed fungal materials, with caveats (Table 2). The DNA barcoding techniques used in this study helped to identify recent changes in systematics and advances in taxonomic nomenclature that are not always apparent to the consumers, growers, and producers of mushrooms. At best, in samples analyzed from capsules containing mushroom powders, the names were validated only to the genus or family level. Such fungal barcoding efforts could be used to ensure that dietary supplements containing fungal materials are properly labeled, particularly if this validation step is taken before excessive processing that could deteriorate genomic DNA. After surveying several products, there is an apparent need in the industry to update practices and ensure labeling is consistent with updated peer reviewed Latin names. This would be consistent with good manufacturing practices after all, and we strive to set new/robust standards for current practices. While ten years ago there may have not been a choice or the tools to drive such clarity,

particularly with fungi, today these tools are available to resolve taxonomic debates. Companies could use these modern molecular techniques to validate the materials in their products.

5. Summary and conclusions

In this study, we have provided aspects on how ITS barcoding can be used for the identification of fungal samples used in dietary supplements. By using such techniques, we envision that dietary supplement manufacturing companies could demonstrate the accuracy of their labeled ingredients for products that contain fungi. These same tools can be used as a means of ingredient validation, well before the final products are produced, which can be particularly valuable for gross materials that are derived from fungal cultures that lack clear morphological characteristics. Barcoding methods highlighted here could ensure the industry of product reliability, thereby ensuring both consumer safety and product integrity. Even when morphology can be discerned, for example in culinary mushrooms, we have revealed that a sample labeled as *B. edulis* and sold in a U.S. grocery store was actually a new species that was recently reported from grocery stores in the UK. We have also demonstrated that some fungal containing products sold commercially as dietary supplement are not entirely accurate in terms of the scientific names that were displayed on the product label. Perhaps more importantly, we have highlighted the pros and cons of obtaining genomic DNA from processed samples, as well as, the pros and cons of the ITS region as a barcode marker in fungi. In short, and with several caveats noted throughout, the ITS data were instrumental in assigning that the proper fungal materials were being utilized. There is a Chinese proverb that “the beginning of wisdom is to call things by their proper name” (Kroll, Shaw, & Oberlies, 2007). Indeed, knowing that the proper Latin binomial is going into the product is a start. We envision that this study will set some standards in place for the mushroom dietary supplement community to barcode fungal samples for accuracy of scientific names via ITS barcoding, and that this study could serve as a platform to build an ITS database of fungal sequences used in mushroom dietary supplements. The knowledge obtained via ITS fungal barcoding can thus be used as a partial solution for product certification.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2016.07.052>.

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