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Assessment of the DNA Quality in Mushroom Specimens: a Recovery of the Whole ITS Sequence from Fragmented DNA of the Type Specimen[1]

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Abstract DNA from four type specimens of mushrooms (Agaricales, Basidiomycota, Fungi) stored in the herbarium of National Museum of Nature and Science since 1950's were extracted and PCR amplification was attempted. Because the whole ITS region (ca. 650 bp) could not be amplified, DNA were assumed to be highly degraded to less than 600 bp. To assess the degree of degradation, we used a microfluidics-based platform. This revealed that the most frequently present fragments were less than 230 bp long. Based on this result, we have designed six new primers to specifically amplify approximately 200–400 bp products of *Amanita imazekii*. A total of 8 contigs were assembled to successfully reconstruct the whole ITS region of the species, and the sequence recovered by this approach had a 100% match with the reference sequence.

Key words: *Amanita*, DNA barcoding, fumigation, holotype, old specimens.

Introduction

As pointed out by many researchers, numerous specimens stored in herbaria are great source of genetic materials (Bruns *et al.*, 1990; Taylor and Swann, 1994; Drábková *et al.*, 2002; Erkens *et al.*, 2008; Dentinger *et al.*, 2010). Undoubtedly, type specimens, especially holotypes, are the most important specimens for solving taxonomic issues. If DNA data from the type specimens become available, we can have a direct link between species names and sequence data, as shown by Nagy *et al.* (2011). Furthermore, herbarium can potentially host a large number of presently unknown or unrepresented lineages. Brock *et al.* (2008) estimated that ca. 70% of the taxonomic diversity in herbarium is not yet represented in the public database.

When we deal with the type specimens, some difficulties include the ages of specimens and poor quality of DNA. Although the ages of specimens and degrees of degradation in DNA are not strictly correlated (Drábková *et al.*, 2002; Leino *et al.*, 2009), DNA in specimens degrades with time (Hummel and Herrmann, 1994; Erkens *et al.*, 2008). This process is unavoidable because DNA molecules degrade as a result of hydrolysis, alkylation, and oxidation even under an ideal condition, i.e., constant low temperature (Willerslev and Cooper, 2005). Therefore to rescue high quality DNA in type specimens, DNA should be extracted as soon as possible or small pieces of specimens should be stored under more ideal conditions for future DNA studies.

It is also claimed that the quality of DNA depends on how specimens were collected, prepared, and maintained (Bruns *et al.*, 1990; Drábková *et al.*, 2002; Leino *et al.*, 2009). For exam-

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ple, previous studies generally agree that temperature and time for dying specimens are two critical factors for preserving quality DNA (Pyle and Adams, 1989; Bruns *et al.*, 1990; Drábková *et al.*, 2002; Blanco *et al.*, 2006; Erkens *et al.*, 2008). Although drying by low heat (ca. 42℃ or less) is often preferred to preserve high quality DNA (Pyle and Adams, 1989; Bruns *et al.*, 1990; Taylor and Swann, 1994; Johnson *et al.*, 2003; Blanco *et al.*, 2006; Nagy, 2010), we have reported, by comparing the DNA quality from phylogenetically distantly related mushroom species, that drying temperature does not significantly affect the DNA quality for subsequent studies (Hosaka and Uno, 2011).

More importantly, many herbarium specimens have been fumigated by a variety of chemicals to prevent insect and fungal damage (Bridson and Forman, 1998; Whitten *et al.*, 1999; Kigawa *et al.*, 2003). Many widely available fumigants are known to highly efficiently degrade DNA, among which the mixture of methyl bromide and ethylene oxide is demonstrated to be one of the worst in terms of DNA degradation (Kigawa *et al.*, 2003). Ideally, all specimens should be treated by fumigants not affecting DNA, such as sulphuryl fluoride (Whitten *et al.*, 1999) or not fumigated at all, but many historically important specimens, especially the type specimens, have already been suffered from such fumigants for a long time.

Our research group has initiated a project of DNA barcoding all type specimens in our fungal herbarium. To achieve this goal, however, we at least need to obtain sequences of the whole ITS region (ca. 600–700 bp), which is now considered an official DNA barcode region for fungi (Schoch *et al.*, 2012). Since we have experienced difficulties amplifying the regions longer than 600 bp from old (ca. 10 year or older) specimens stored in our herbarium (Hosaka and Uno, 2011), we assumed that DNA in such specimens are highly degraded due to the ages and fumigation. However, we have not assessed the sizes of DNA fragments so far.

Because repeated destructions of specimens for molecular studies should be avoided, we have initiated DNA extraction from taxa with large and fleshy fruit bodies. Among them, a genus *Amanita* contains more than 900 species worldwide (Tulloss, 2005) with great diversity in East Asia (Zhang *et al.*, 2004). Furthermore, the genus has been a subject of molecular phylogenetic studies (Oda *et al.*, 1999, 2004; Zhang *et al.*, 2004). Our herbarium possesses ca. 20 type specimens of the genus from various ages (1950's to 2000's) and therefore we have selected the specimen collected relatively recently (1990's) as a case study to assess the DNA quality.

In addition to a standard PCR using general primers, we also used the Agilent Bioanalyzer (Palo Alto, CA, USA) to visualize the quantity of DNA in each fragment size. This technology uses a microfluidics-based platform for sizing, quantification and quality control of DNA (http://www.home.agilent.com) and it has a potential of further application, such as evaluating quality and quantity of total RNA (Miller *et al.*, 2004). By using this method, we can assess degrees of DNA fragmentation more objectively than using a standard gel electrophoresis. We here report the results of Bioanalyzer assay and a recovery of the whole ITS sequence from fragmented genomic DNA of the type specimen.

Materials and Methods

Type specimen used in this study

The holotype of *Amanita imazekii* T. Oda, C. Tanaka & Tsuda (TNS-F-101519) was collected in 1996 and has been stored in the fungal herbarium of National Museum of Nature and Science (Fig. 1). It was described as a new species in 2001 (Oda *et al.*, 2001) but the whole ITS region had already been sequenced and been deposited in GenBank under the accession number AB038764 (submitted on February 23, 2000). The sequence was obtained before the specimen was officially registered at the TNS herbarium. To compare the quality of genomic DNA, three additional type specimens collected and stored in


TNS-F-101519

MYCOFLORA OF JAPAN HOLOTYPE

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Specimen No. LEM 960277
Specimen Name *Amanita imazekii* Oda, Tanaka & Tsuda

和名 ミヤマタマゴタケ
Date 1996.9.12
Locality 岐阜県大野郡高根村日和田高原
Hiwada-kogen, Takane-mura, Ohno-gun, Gifu Pref.

Coll. T. ODA 小田 貴志
Det. T. ODA 小田 貴志

Fig. 1. Label of the holotype of *Amanita imazekii* housed at the TNS herbarium.

the TNS herbarium from earlier times (1950's to 1980's) were used as well. These specimens include *Amanita pseudoporphyria* (TNS-F-237281, collected in 1956), *Lyophyllum sykosporum* (TNS-F-237381, collected in 1986), and *Russula subnigricans* (TNS-F-237524, collected in 1954).

DNA extraction

DNA was extracted from the gill tissues stored in DMSO buffer (Seutin *et al.*, 1991; Hosaka, 2009) overnight. Tissues were first ground under liquid nitrogen using a mortar and pestle. Ground tissues were then transferred to new 1.5 ml tubes using clean spatulas, and CTAB buffer was added. DNA was extracted using the modified CTAB extraction protocol (Doyle and Doyle, 1987) followed by glass milk purification methods as summarized by Hosaka (2009) and Hosaka and Castellano (2008). Briefly, ground samples were incubated in CTAB buffer at 65°C for 1 hour, and proteins were removed using the mixture of chloroform: isoamylalcohol (24: 1). The materials were further purified using 6M sodium iodine buffer with glass milk, washed with ethanol/buffer solution, and finally eluted in 100 μl of TE buffer.

Assessment of DNA fragmentation using the Bioanalyzer

The sizes of DNA were determined by microcapillary electrophoresis using the Agilent Bioanalyzer (Agilent Technology, Palo Alto, CA, USA). Samples (1 μl) of genomic DNA were loaded into each well of High Sensitivity DNA LabChip for analysis. The obtained data were analyzed using the Expert Software 2100 (Agilent Technologies, Palo Alto, CA). The Bioanalyzer assay was processed and analyzed following the manufacturer's instructions.

PCR and sequencing

DNA sequence data were obtained from the internal transcribed spacer regions (ITS) of the nuclear ribosomal DNA. For amplifying the whole ITS region, the primer combination of ITS5 and ITS4 (White *et al.,* 1990) was used. Because the amplification of the whole ITS region was difficult for this sample, we have designed six new primers located internally between ITS5 and ITS4 (Fig. 4, Table 1). As mentioned above, the ITS sequence of the same specimen has already been deposited in GenBank (accession number = AB038764), which was used as a reference sequence for primer design. Primers were designed using the Primer 3 ver. 0.4.0. (http://primer3.sourceforge.net/) to amplify approximately 200–400 bp products.

PCR reactions were carried out using 20 μl reaction volumes each containing: 1 μl genomic DNA, 1 μl dNTP, (4 mM), 1 μl of each primer (8 μM), 0.5 units of Taq polymerase (TaKaRa, Tokyo, Japan), 2 μl $MgCl_2$ (25 mM), 2 μl Bovine Serum Albumin (BSA). Cycling parameters were 1 cycle of 94°C for 3 min, 30 cycles of 94°C for 1 min, 51°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 15 min. PCR products were electrophoresed in 1% agarose gels stained with ethidium bromide and visualized under UV light. When amplification bands were confirmed, PCR products were then purified using the ExoSap-IT (Millipore, Molsheim, France) and directly sequenced using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Norwalk, CT, USA), following the manufacturer's instructions. The obtained sequences were edited and assembled along with the reference sequence from the type specimen (accession number AB038764) using ATGC Ver. 7.0.2

(Genetyx Corporation, Tokyo, Japan).

Results

Type specimen used in this study

Visual inspection revealed that the specimen had no severe insect damage, except the one caused prior to collection (Fig. 2). No fungal growth, which can make DNA extraction problematic, was observed, either (Fig. 2). Major microscopic features such as basidiospores, basidia, and hyphal structures were well preserved.

DNA extraction and initial PCR attempt

By using general primers for the ITS region (ITS5 and ITS4), no visible bands were obtained. The results were the same after several attempts of DNA extraction. We have purified the PCR products for direct sequencing, but no informative sequences, including the ones from contaminants, were obtained.

Assessment of DNA fragmentation using the Bioanalyzer

Electropherogram of four DNA samples from the holotype specimens (Fig. 3) revealed that DNA from *Amanita imazekii* was fragmented, but the quality (in terms of degrees of fragmentation) and quantity of its DNA was higher than the other three DNA samples, which were from the specimens collected in earlier times (1954 to 1986). The analysis using the Expert Software 2100 (Agilent Technologies, Palo Alto, CA) indicated that the peak of the DNA from *Amanita imazekii* was located at 229 bp. On the other hand, the other three DNA samples had peaks at ca. 100 bp. The average size of DNA fragments from *Amanita imazekii*, when measured between 50 bp to 1000 bp, was ca. 363 bp.

PCR using newly designed primers and sequencing

Our attempt to amplify approximately 200–400 bp products using the combination of existing (ITS5 and ITS4) and newly designed primers (Amaima-F1, F2, F3, R2, R3, R4) (Fig. 4, Table 1) was successful. The amplified bands were typically bright and clear, and direct sequencing after purification was mostly successful. A total of 8 contigs were assembled to reconstruct the whole ITS region. The sequence recovered by this approach had a 100% match with the reference sequence from the same specimen (acces-

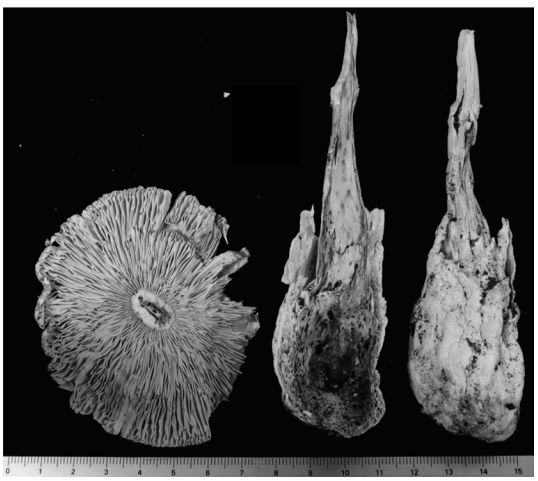

Fig. 2. Type specimen of *Amanita imazekii*. The scale at the bottom represents 15 cm.

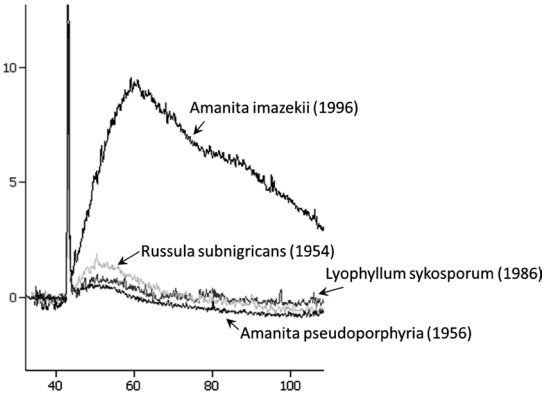

Fig. 3. The result output of the Bioanalyzer assay. Taxon names of each DNA samples are followed by the year of collection in parentheses. The numbers of the X axis indicate the relative length of DNA fragments, not the actual base pairs. The numbers of the Y axis indicate the relative quantity of DNA fragments. The sharp peak at the left side is a size marker (35 bp).

sion number = AB038764).

Discussion

Despite the fact that macro- and microscopic characters are well maintained (Fig. 2), no PCR bands were obtained using general primers (ITS5 and ITS4) to amplify ca. 650 bp. This is probably not due to insect and/or fungal infestation because no bands or sequences from contaminants were obtained, and the PCR using newly designed internal primers (Fig. 4, Table 1) successfully amplified the target regions. The longest PCR products we obtained were ca. 400 bp and the amplification of fragments shorter than 400 bp had a 100% success rate. This is consistent with the result of the Bioanalyzer assay (Fig. 3), which indicated that the most frequently present fragments were ca. 230 bp. The other three type specimens we have tested all possessed highly fragmented DNA because the peaks of electropherogram were located at ca. 100 bp. The older ages and therefore more repeated dose of fumigations must have contributed to this phenomenon.

The DNA of the holotype of *Amanita imazekii*, however, was not free from fragmentation, either. Although we do not have a detailed lab record, the amplification and sequencing of the whole ITS region was probably not a problem when the reference sequence (accession number = AB038764) was submitted to GenBank in 2000. This strongly indicates that fragmentation of DNA occurred after the specimen was relocated to the TNS herbarium.

Our herbarium (TNS) had been treated by methyl bromide until relatively recently. The chemical had been applied twice a year until 2003 or so. The mixture of methyl bromide and ethylene oxide or other chemicals, such as methyl iodide, can more severely degrade DNA than methyl bromide alone (Kigawa *et al.*, 2003), but the moderate degradation observed in DNA of *Amanita imazekii* indicates that a few years doses of methyl bromide have apparently negative impacts. The TNS herbarium has been treated by sulphuryl fluoride (Vikane®) since 2004, and we assume that DNA degradation by fumigation after 2004 has little effect because this chemical is claimed not to affect the DNA molecules (Whitten *et al.*, 1999; Kigawa *et al.*, 2003).

We tested using only one DNA extraction protocol, and this may bias our results. A variety of DNA extraction methods have been proposed (Bruns *et al.*, 1990; Drábková *et al.*, 2002; Dentinger *et al.*, 2010; Izumitsu *et al.*, 2012), and some methods may be more efficient for extract-

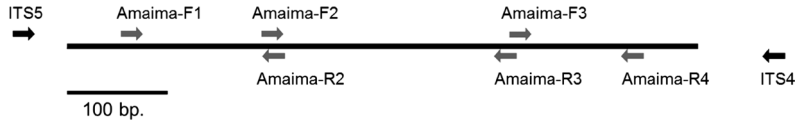

Fig. 4. PCR primer map of the ITS region. The PCR primers designed specifically for this study are depicted as gray arrows.

Table 1. Primers used in this study.

Primer	5′-sequence-3′	Source
Amaima-F1	ATTGGAATGAAAACTCTGG	This study
Amaima-F2	TTGAATGTTATTGGCAAGGT	This study
Amaima-F3	TTATTGCTGGCCTTTTTGGT	This study
Amaima-R2	TGACCTTGCCAATAACATTC	This study
Amaima-R3	AGCAATAATTCCCAATATCC	This study
Amaima-R4	GTCAATTTGTCAACACGGCT	This study
ITS5	GGAAGTAAAAGTCGTAACAAGG	White *et al.* (1990)
ITS4	TCCTCCGCTTATTGATATGC	White *et al.* (1990)

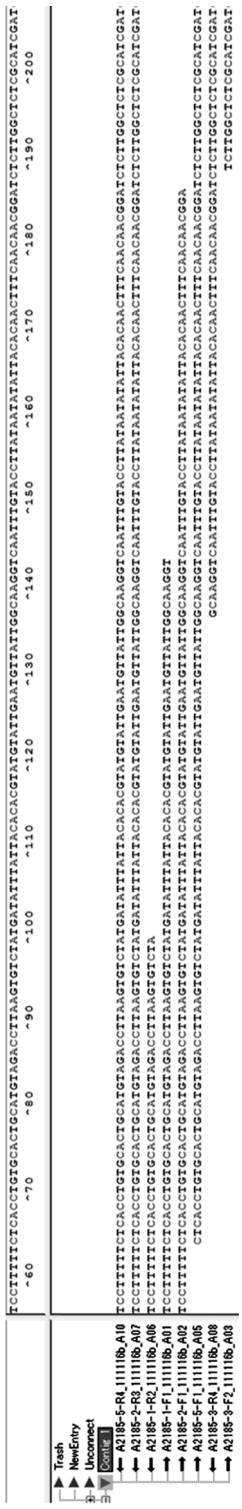

Fig. 5. A portion of the ITS region reconstructed from contigs shorter than 400 bp.

ing highly degraded DNA because DNA fragments shorter than 200 bp do not efficiently bind to silica particles in glass milk. Nevertheless, a failure of PCR amplification of 600 bp products and the results of the Bioanalyzer assay (Fig. 3) are consistent with our hypothesis that DNA from four type specimens we tested are all degraded.

A failure of PCR amplification can also be caused by interstrand crosslinks by alkylation or intermolecular crosslinks by Maillard reaction (Willerslev and Cooper, 2005). Crosslinks are not the direct cause of DNA degradation, but such crosslinks may decrease the efficiency of DNA extraction, or cause fragmentation of DNA during the extraction procedure. Oxidative and hydrolytic modification of nucleotides, especially CG→TA transitions, may also cause the PCR failure (Willerslev and Cooper, 2005), but our results of contig assembly (Fig. 5) indicate such substitutions did not occur at the ITS region.

The whole ITS region of *Amanita imazekii* was successfully recovered because the degree of degradation was not as severe and the reference sequence was available. This is probably not the case for many other type specimens, which were collected in earlier times and therefore possess highly degraded DNA. To recover long fragments of DNA from such specimens, we have to deal with even shorter fragments by using "primer walking" (Fraser and Fleischmann, 1997) or a next-generation sequencing approach.

A problem of DNA degradation in historically important specimens is common to the herbaria around the world. For example, PCR amplification of some specimens stored in Chinese herbaria from the late 1980's was not possible (Hosaka *et al.*, 2010). Because DNA in specimens degrades with time (Hummel and Herrmann, 1994; Erkens *et al.*, 2008), we strongly suggest that DNA from historically important specimens should be extracted as soon as possible, or small pieces of specimens should be stored under more ideal conditions for future DNA studies. One way of preventing further degradation of DNA is a cryopreservation in liquid

nitrogen or ultra-deep freezer at −80°C (Nagy, 2010). If such facilities are not available, use of several organic solvents, such as acetone (Fukatsu, 1999), or DNA preservation buffers (Rogstad, 1992; Laulier *et al.*, 1995; Dawson *et al.*, 1998; Johnson *et al.*, 2003; Nagy, 2010) should be considered.

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