Discrimination between edible and poisonous mushrooms among Japanese *Entoloma sarcopum* and related species based on phylogenetic analysis and insertion/deletion patterns of nucleotide sequences of the cytochrome oxidase 1 gene

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(Received 19 July 2019, accepted 28 February 2020; J-STAGE Advance published date: 30 July 2020)

*Entoloma sarcopum* is widely known as an edible mushroom but appears morphologically similar to the poisonous mushrooms *E. rhodopolium* sensu lato (s. l.) and *E. sinuatum* s. l. Many cases of food poisoning caused by eating these poisonous mushrooms occur each year (Suzuki et al., 1987; Nagasawa and Hongo, 1999; Suzuki and Watanabe, 2005) in Japan. Particularly, *E. rhodopolium* s. l. caused 87 cases from 2006 to 2015 because it was mistaken for *E. sarcopum*. These three species are closely related and are morphologically similar. A recent study led to their reclassification based on both morphological and molecular characteristics as sensu stricto (s. str.) species (Kondo et al., 2017). The authors indicated that *Entoloma* species in Japan are clustered in a complex of *E. rhodopolium*-related species including poisonous species of *E. lacus*, *E. pseudorhodopolium* and *E. subrhodopolium*, and edible *E. sarcopum*. Thus, identification based on morphological characteristics alone often leads to mis-identification.

Recently, analysis based on the nucleotide sequences of genes has been used to identify fungi. In fungal studies including those of mushrooms, the internal transcribed spacer (ITS) 1 region in the rRNA gene (rDNA) cluster is widely used as a genetic marker (Dentinger et al., 2010; Schoch et al., 2012; Parnmen et al., 2016). However, there are cases in which mushrooms cannot be identi-
fied by molecular phylogenetic analysis because of the low sequence diversity in the ITS1 region among closely related species. For molecular phylogenetic analysis of various organisms, the mitochondrial cytochrome oxidase (CO1) gene is widely used as a genetic marker. This gene has been used to evaluate relationships among fungi in recent work (Seifert et al., 2007). In this study, we performed phylogenetic analyses based on the nucleotide sequences of both the ITS regions and the CO1 gene to evaluate these two loci as genetic markers for discrimination between edible and poisonous mushrooms among Japanese *E. sarcopum* and other poisonous species.

**MATERIALS AND METHODS**

**Tested samples and their classification** Samples were collected in Hokkaido, Iwate, Yamagata, Fukushima, Tochigi, Gunma, Niigata, Tokyo, Yamanashi and Ishikawa prefectures in Japan. First, a total of 49 samples were identified based on their morphological characteristics as described below and were considered where possible as s. l. species: *E. sarcopum* and *E. rhodopolium* (Table 1). Second, as described later, rDNA cluster sequences of the 49 samples were determined in this study or downloaded from GenBank, and we performed phylogenetic analyses for identification as s. str. species using 11 entries downloaded from GenBank in an ingroup, including holotype strains of three new species derived from poisonous *E. rhodopolium* s. l. in Japan, described by Kondo et al. (2017) as *E. subrhodopolium* (GenBank accession no. LC088033), *E. lacus* (LC088043, LC088045, LC088046 and LC088049–LC088052) and *E. pseudorhodopolium* (LC088042). In addition to these, we added three entries of two species in an ingroup, namely *E. whiteae* (LC088034 and KC710084) and *E. sericatum* (LN850460), because they are indicated as species closely related to *E. rhodopolium* s. l. by Kondo et al. (2017) although we have no information about their edibility.

**Morphological observation** To distinguish the edible species *E. sarcopum* from the poisonous species *E. rhodopolium* s. l. is very important in food hygiene. Therefore, the morphological characteristics of tested samples in this study were compared with descriptions of *E. sarcopum* and *E. rhodopolium* (Imazeki and Hongo, 1987). The fruiting body was evaluated by direct observation of the pileus color, form, lamella and stripe. Furthermore, the lamella was examined under a microscope to determine the surface pattern of the three-dimensional structure, size of the spores, and presence of cystidia and basidia based on a method described previously (Largent, 1986).

**Nucleotide sequencing** DNA was extracted from the fruiting body with a DNeasy Plant Mini Kit (QIAGEN) and Maxwell RSC Plant DNA Kit (Promega). PCR primers for amplifying CO1 genes of *Entoloma* species were designed in this study (Table 2), derived from nucleotide sequences from Dentinger et al. (2011). The ITS1, 5.8S and ITS2 regions in the rDNA cluster were amplified and sequenced with primer pairs as previously described (Gardes and Bruns, 1993). The PCR was performed with KOD FX Neo (Toyobo) according to the manufacturer’s instructions. The amplification cycle was 94 °C for 30 s, 60 °C for 40 s and 68 °C for 50 s for the rDNA cluster region, and 94 °C for 30 s, 48 °C for 40 s and 68 °C for 2 min for the CO1 gene. The PCR products were dye-labeled and sequenced using a BigDye Terminator v. 3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and ABI 3730xl analyzer (Thermo Fisher Scientific). The sequences were assembled using ATGC (Genetyx).

**Phylogenetic analysis** Phylogenetic analyses of nucleotide sequences determined in this study were conducted in MEGA7 (Kumar et al., 2016) with sequences downloaded from GenBank as outgroups (GenBank accession numbers for rDNA cluster: JN029433 *E. clypeatum*, KC581296 *E. conferendum*, AF335449 *E. nitidum*, AY228348 *Citopilus prunulus*, FJ039635 *Cortinarius allatius*; and for CO1: JN029430 *E. sericionitidum*, JN029426 *Entoloma sp.*, JN029427 *Entoloma sp.*, JN029428 *Entoloma sp.*, JN029429 *Entoloma sp.*, JN029434 *Entoloma sp.*, JN029379 *Boletus edulis*, JN029414 *Clitocybe robusta*, JN029425 *Cortinarius cf. violaceus*, JN029450 *Hygrocybe persistens*, JN029490 *Pluteus cervinus*, HM625879 *Tricholoma auratum*). Evolutionary history was inferred using the neighbor-joining (NJ) method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown on the trees as bootstrap values (BPs) next to the branches, for BPs of 80% or more. Evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004) and are in units of the number of base substitutions per site. Rate variation among sites was modeled with a gamma distribution.

**Comparison of the insertion/deletion pattern in CO1 nucleotide sequences** The sequences determined in this study were aligned with sequences downloaded from the database described above and are shown in Table 1, using MEGA7 to detect highly similar regions of nucleotide sequences. The sequences in these regions were compared with the *Russula compacta* mitochondrial complete genome (NC_037773) to identify exons and introns.

**RESULTS**

In morphological observation, a total of 49 samples
## Table 1. *Entoloma* strains identified in this study

| Strain No. | Species based on morphology (sensu lato) | Species based on rDNA nucleotide sequences (sensu stricto) | Source of nucleotide sequence rDNA CO1 |
|------------|------------------------------------------|------------------------------------------------------------|---------------------------------------|
| KUB-1      | *Entoloma rhodopolium*                   | *Entoloma subrhodopolium*                                   | DL¹ (LC088033) This study             |
| KUB-2      | *Entoloma rhodopolium*                   | *Entoloma whiteae*                                           | DL (LC088034) This study             |
| KUB-A      | *Entoloma rhodopolium*                   | *Entoloma subrhodopolium*                                   | This study This study                 |
| KUB-B      | *Entoloma sp.*                           | Unidentified²                                                | This study This study                 |
| KUB-C      | *Entoloma sp.*                           | *Entoloma whiteae*                                           | This study This study                 |
| KUB-102    | *Entoloma sp.*                           | *Entoloma pseudorhodopolium*                                 | DL (LC088042) Not sequenced³         |
| KUB-103    | *Entoloma sp.*                           | *Entoloma sericatum*                                         | This study This study                 |
| KUB-104    | *Entoloma sp.*                           | *Entoloma lacus*                                             | DL (LC088043) This study             |
| KUB-106    | *Entoloma sp.*                           | *Entoloma lacus*                                             | DL (LC088045) This study             |
| KUB-107    | *Entoloma sp.*                           | *Entoloma lacus*                                             | DL (LC088046) This study             |
| KUB-110    | *Entoloma sp.*                           | *Entoloma lacus*                                             | DL (LC088049) This study             |
| KUB-111    | *Entoloma sp.*                           | *Entoloma lacus*                                             | DL (LC088050) This study             |
| KUB-113    | *Entoloma sp.*                           | *Entoloma lacus*                                             | DL (LC088051) This study             |
| KUB-114    | *Entoloma sp.*                           | *Entoloma lacus*                                             | DL (LC088052) This study             |
| KUB-203    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |
| KUB-205    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | DL (LC088067) This study             |
| AKK-1      | *Entoloma sp.*                           | *Entoloma subrhodopolium*                                   | This study This study                 |
| AKK-2      | *Entoloma sp.*                           | Unidentified                                                | This study This study                 |
| AKK-3      | *Entoloma sp.*                           | Unidentified                                                | This study This study                 |
| AKK-4      | *Entoloma sp.*                           | Unidentified                                                | This study This study                 |
| AKK-5      | *Entoloma rhodopolium*                   | *Entoloma pseudorhodopolium*                                 | This study This study                 |
| AKK-7      | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |
| AKK-8      | *Entoloma rhodopolium*                   | *Entoloma subrhodopolium*                                   | This study This study                 |
| AKK-9      | *Entoloma rhodopolium*                   | *Entoloma pseudorhodopolium*                                 | This study This study                 |
| AKK-11     | *Entoloma rhodopolium*                   | *Entoloma subrhodopolium*                                   | This study This study                 |
| AKK-12     | *Entoloma rhodopolium*                   | *Entoloma subrhodopolium*                                   | This study This study                 |
| AKK-13     | *Entoloma rhodopolium*                   | *Entoloma subrhodopolium*                                   | This study This study                 |
| AKK-14     | *Entoloma rhodopolium*                   | *Entoloma subrhodopolium*                                   | This study This study                 |
| AKK-16     | *Entoloma rhodopolium*                   | *Entoloma subrhodopolium*                                   | This study This study                 |
| AKK-17     | *Entoloma rhodopolium*                   | *Entoloma subrhodopolium*                                   | This study This study                 |
| AKK-22     | *Entoloma rhodopolium*                   | Unidentified                                                | This study This study                 |
| AKK-101    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |
| AKK-102    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |
| AKK-103    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |
| AKK-106    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |
| AKK-107    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |
| AKK-108    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study Not sequenced              |
| AKK-111    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |
| AKK-112    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study Not sequenced              |
| AKK-113    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |
| AKK-202    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |
| AKK-203    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |
| AKK-204    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |
| AKK-205    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |
| AKK-206    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |
| AKK-207    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |
| AKK-208    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |
| AKK-209    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |
| AKK-213    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |
| AKK-225    | *Entoloma sarcopum*                      | *Entoloma sarcopum*                                          | This study This study                 |

¹Downloaded from NCBI database.
²Unidentified in the rDNA tree in this study.
³The sequence was not determined in this study.
Table 2. Primers used for PCR and sequencing in this study

| Primer     | Primer sequence (5′→3′)      | Target species (sensu lato)     |
|------------|-------------------------------|---------------------------------|
| Entoloma-CO1-F1 | TTACAAGGTGATCATCAATT | Entoloma rhodopolium          |
| Entoloma-CO1-R1 | TTTCTATCTGTAAGTAACT       | Entoloma rhodopolium          |
| Entoloma-CO1-F2 | GTATTAAAATTTCTATCTGTAAG  | Entoloma sarcopum             |
| Entoloma-CO1-R2 | TTACAAGGTGATCATCAA       | Entoloma sarcopum             |

Fig. 1. Phylogenetic trees based on nucleotide sequences of the genus *Entoloma*. (A) shows a tree of the rDNA cluster region (564 bp), and (B) a tree of the CO1 gene (362 bp). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown as bootstrap values (BPs) next to the branches, with BP values of 88% or more marked on each tree. The OTUs in black boxes are samples unidentified in tree (A) but identified in tree (B).

were evaluated including 13 of poisonous *E. rhodopolium* s. l., 22 of edible *E. sarcopum* and 15 of unidentified *Entoloma* sp. We determined the nucleotide sequences of the rDNA cluster and the CO1 gene with these samples (GenBank accession numbers: LC497670–LC497707 for the rDNA cluster, and LC497708–LC497756 for CO1). In
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![Diagram of phylogenetic tree and insertion/deletion patterns](image)

**Fig. 2.** Comparison between a simplified tree and insertion/deletion patterns found in the CO1 gene. Left, the phylogenetic tree of *Entoloma* species based on partial nucleotide sequences of the CO1 gene and derived from Fig. 1B. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown as bootstrap values (BPs) next to the branches, for BPs of 94% or more. Right, insertion/deletion patterns in partial nucleotide sequences of the CO1 gene. Four groups were defined by insertion/deletion in the CO1 gene, indicating the length of groups A, B, C and D as about 1,200, 500, 3,000 and 1,800 bp, respectively. The four groups shared three highly similar regions of nucleotide sequences in exons 1, 2 and 3. Primer annealing positions are indicated by arrows.

A previous study of a wide area in Japan, no sample was definitively identified as *E. sinuatum* (Kondo et al., 2017); this species has been reported to have a broad range in Europe, and is poisonous. No samples were morphologically identified as *E. sinuatum* s. l. in this study. Our NJ tree based on the nucleotide sequences of the rDNA cluster was divided into eight lineages supported by bootstrap values of at least 88% (Fig. 1A). However, five samples, AKK-2, AKK-3, AKK-4, AKK-22 and KUB-B, did not form a monophyletic group with any entry sequence in GenBank; therefore, these five samples were not identified as s. str. species. In contrast, our NJ tree based on nucleotide sequences of the CO1 gene was divided into five clearly differentiated lineages supported by high BPs of at least 94% (Fig. 1B). Four of the unidentified samples in our rDNA tree, AKK-2, AKK-3, AKK-4 and KUB-B, belonged to the *E. subrhodopolium/E. pseudorhodopolium* clade in our CO1 tree, supported by a BP of 97%.

In the CO1 gene of fungi, insertions and deletions have frequently been reported (Seifert et al., 2007; Dentinger et al., 2011). In this study, alignment of our partial nucleotide sequences of the CO1 gene revealed four patterns amplified by our PCR primers (groups A–D; Fig. 2). We identified exons and introns by an alignment with the complete nucleotide sequence of the CO1 gene of *Russula compacta* (NC_037773). Our results indicated that all insertion/deletion sequences were in introns, with highly conserved nucleotide sequences in exons (data not shown).

The four groups were defined by the insertion pattern between exons, indicating the nucleotide length of groups A, B, C and D as about 1,200, 500, 3,000 and 1,800 bp, respectively. The four groups shared three highly similar regions of nucleotide sequences in exons 1, 2 and 3. The four groups were observed in a simplified CO1 tree to compare the patterns among lineages of *Entoloma* species (Fig. 2). The appearance of each of the four groups was completely consistent with the phylogenetic monophyly in our CO1 tree. Group A appeared only in the lineage of *E. subrhodopolium/pseudorhodopolium*. Group B appeared only in the *E. aff. sinuatum* and *E. whiteae* lineages. Group C appeared only in the *E. lacus* lineage. Group D appeared only in the *E. sarcopum* lineage. Introns were found in three lineages of group A, C and D between three exons, and no introns were found in the lineage of *E. whiteae* and *E. aff. sinuatum* in group B (Fig. 2).

**DISCUSSION**

Our results indicated that two lineages of *E. subrhodopolium* and *E. pseudorhodopolium* could not be distin-
guished from each other using either the CO1 tree (Fig. 1B) or the nucleotide insertion/deletion pattern in the CO1 gene (Fig. 2). Five samples were not identified in the rDNA tree because they did not form a monophyletic group with reference sequences from the database (Fig. 1A). However, four of these samples, AKK-2, AKK-3, AKK-4 and KUB-B, were classified as E. subrhodopolium or E. pseudorhodopolium by both our CO1 tree and the CO1 insertion/deletion pattern. Thus, these four samples are considered as poisonous species. Furthermore, our CO1 tree also indicated that the fifth sample that was unidentified in the rDNA tree, AKK-22, belongs to the E. whiteae clade (Fig. 1B). Because E. whiteae has been found only in North America (Morgado et al., 2013), our assignment of AKK-22 here represents the first suggestion that this species also occurs in Japan, although it is unclear from previous studies whether E. whiteae is edible or poisonous. However, since Morgado et al. (2013) reported that E. whiteae is closely related to a well-known poisonous species, E. sinuatum, we inferred that AKK-22/E. whiteae may be poisonous. With the exception of AKK-22, our results indicated that CO1 nucleotide sequence analysis can be used to distinguish clearly between edible and poisonous species of Japanese Entoloma.

One of the main reasons that the five samples of AKK-2, AKK-3, AKK-4, AKK-22 and KUB-B were not identified by our rDNA tree (Fig. 1A) was the high divergence in nucleotide sequences of ITS regions in E. subrhodopolium and E. pseudorhodopolium. Only two reference sequences (KUB-1 and KUB-102) were used in our rDNA tree, which may not have been enough to match the nucleotide sequences of these five samples. In fungi, including mushrooms, the high degree of genetic diversity within a species has been reported previously (Co-David et al., 2009; Morgado et al., 2013; Kokkonen, 2015). Maeta et al. (2008) described rapid species identification of cooked poisonous mushrooms by real-time PCR with ITS regions. However, because their data set did not include enough genetic diversity among poisonous Entoloma species in Japan, their method may not be able to detect several populations of poisonous Japanese Entoloma.

We also suspect that the discrepancy between CO1 and rDNA tree topologies for these five samples is caused by introgression in the mitochondrial CO1 gene because the majority of the reported cases of introgression in animals involve the mitochondrial genome (Arnold, 1993; Seixas et al., 2018). In several fungal species, researchers previously suggested that introgression events occurred in the evolutionary history of the mitochondrial DNA (Fourie et al., 2018). Therefore, it is necessary to analyze more nuclear and mitochondrial genes than the rDNA and CO1 genes.

Our rDNA tree strongly suggested (BP, 99%) that the sample KUB-103 is E. sericatum, whereas the CO1 tree (BP, 98%) and CO1 insertion/deletion pattern identified KUB-103 equally strongly as E. aff. sinuatum. We are thus currently unable to assign KUB-103 to a particular Entoloma species. However, both E. sericatum and E. sinuatum are known to be distributed across Europe and North America, and a previous study did not mention the occurrence of either species in Japan (Kondo et al., 2017). This is therefore the first report of the possible distribution of the poisonous species E. sericatum or E. sinuatum in Japan.

Dentinger et al. (2011) reported that insertion/deletion patterns were non-conservative within a genus of mushrooms. For the species of Entoloma tested in this study, insertion/deletion regions in each of lineage in Fig. 2 were conserved as lineage-specific patterns, not as a genus-specific pattern. Therefore, our study has reported a new genetic marker that is useful for detection of Japanese poisonous Entoloma. However, to take full advantage of this genetic marker, the CO1 gene, further entries of reference sequences in databases are essential for accurate identification of more species in the future.

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