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RESEARCH ARTICLE

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Mycorrhization of *Quercus* spp. with *Tuber huidongense* and *T. himalayense* Collected in Korea

Ju-Hui Gwon, Hyeok Park and Ahn-Heum Eom

Department of Biology Education, Korea National University of Education, Cheongju, Korea

ABSTRACT

Fungi of the genus *Tuber* are ectomycorrhizal fungi that form a symbiotic relationship mainly with oak and hazel trees. *Tuber* spp. exhibit a highly selective host plant preference; thus, for cultivation purposes it is important to select an appropriate host plant for successful mycorrhization. In addition, as mycorrhizal characteristics differ according to *Tuber* spp., it is necessary to understand the differences in mycorrhizae according to the fungal species. *Tuber huidongense* and *Tuber himalayense* were recently discovered in Korea; therefore, we used spore suspensions from these two species to inoculate two species of oak trees, *Quercus acutissima* and *Quercus dentata*, to compare colonization rates and morphologies of the mycorrhizae. The colonization rates demonstrated that the different *Tuber* spp. favored different host plant species. In addition, unique morphological and anatomical characteristics were observed for *T. huidongense* and *T. himalayense* depending on the host species. These findings can lead to new economically important agricultural activities related to truffle cultivation in Korea.

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1. Introduction

Fungi in the genus *Tuber* (Ascomycota, Pezizales) emit a strong, diverse, and long-lasting aroma and the high value of truffles is largely attributed to their aroma. *Tuber* spp. form ectomycorrhizal relationships with diverse plants, especially oak trees [1]. Different *Tuber* spp. exhibit a highly selective preference for specific host plants [2]. For example, *Tuber gibbosum* and *Tuber oregonense* form a unique symbiotic relationship with Douglas fir (*Pseudotsuga menziesii*) [3], whereas truffles native to Greece form associations with *Quercus coccifera*, *Quercus ilex*, and *Quercus pubescents* [4].

For *Tuber melanosporum*, the efficiency and strength of mycorrhization differ depending on the host plant species, showing the highest colonization rate with *Q. ilex* [4]. Depending on the *Tuber* spp., there are differences in the characteristics of mycorrhizae that form in the same host plant species [5,6]. When comparing the ectomycorrhiza (ECM) of white truffles formed on the same host plant, the anatomical structure and size of the cystidia, the cell wall thickness of the mantle, and type of transition morphology between the epidermoid and angular types differ for each species [5]. In addition, *Tuber* spp. exhibit different mycorrhizal morphologies depending on the host plant species [7].

Although there have been few studies on truffles in Korea, our study was facilitated by the recent discovery of two species of truffles, *Tuber huidongense* and *Tuber himalayense* [8,9]. The purpose of this study was to provide information on the morphology and anatomical characteristics of ECM and identify suitable host plants by comparing colonization rates following inoculation of *T. huidongense* and *T. himalayense* into the host trees *Quercus acutissima* and *Quercus dentata*. *Quercus acutissima* is a common species throughout Korea, whereas *Q. dentata* is a known host species for both *T. huidongense* and *T. himalayense* in Korea.

2. Materials and methods

2.1. Preparation of seedlings

Quercus acutissima acorns were collected from a natural site in Danyang, Korea, and *Q. dentata* acorns were collected from a market in Youngju, Korea. The acorn shells were removed and the seeds were surface-sterilized with 10% sodium hypochlorite (NaClO) for 30 min. The seeds were then placed in a plastic pot (280 mL) with an autoclaved 1:1 mixture of vermiculite and perlite, and cultured in a greenhouse for 6 months (8 h photoperiod, 55 ± 5% relative humidity, and 24 ± 1 °C).

CONTACT Ahn-Heum Eom eomah@knue.ac.kr

2.2. Inoculation of Quercus seedlings and mycorrhizal synthesis

The fruiting bodies of *T. huidongense* and *T. himalayense* were collected from the rhizospheres of *Q. dentata* in Pohang [8] and Danyang [9], respectively. The fruiting bodies were surface-sterilized with 70% ethanol and ground with distilled water using a blender. Six-month-old seedlings of *Q. acutissima* and *Q. dentata* were inoculated near the roots with a 1 mL spore suspension containing 1.5×10^6 spores of *T. huidongense* or *T. himalayense* (determined using a hemocytometer; Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany), and then roots were wrapped with a non-woven fabric. *Tuber huidongense* was inoculated into five seedlings of the two host species (10 seedlings total), whereas *T. himalayense* was inoculated into 10 seedlings of the two host species (20 seedlings total). The inoculated seedlings were transplanted into a plastic pot (280 mL) with an autoclaved 1:1 mixture of vermiculite and perlite; slaked lime was added to maintain the pH at 8. The pots were maintained in a greenhouse for 8 months and watered weekly.

2.3. Molecular identification of ECM

To determine the successful formation of ECM in each inoculated seedling, genomic DNA was extracted from a mycorrhizal root tip using the DNeasy Plant Mini kit (QIAGEN GmbH, Hilden, Germany). The ribosomal DNA internal transcribed spacer (ITS) region was then amplified using the primer pair ITS1F/ITS4 [10]. The nucleotide sequences were analyzed (SolGent Co. Ltd., Daejeon, Korea) and identified using BLAST (https://www.ncbi.nlm.nih.gov/).

2.4. Morphological and anatomical characteristics of ECM

After 8 months of growth, seedlings were harvested and the morphological and anatomical characteristics of the ECM of *T. huidongense* and *T. himalayense* were observed. Morphological characteristics were observed using a dissection microscope (Olympus SZX7, Tokyo, Japan). After cross-sectioning the roots using a cryostat (CM1850, Leica, Heidelberg, Germany), the anatomical characteristics of the ECM were observed and recorded using a light microscope (Axio Imager A1, Carl ZEISS, Oberkochen, Germany).

2.5. Mycorrhizal root colonization rates

After 8 months of growth, seedlings were carefully removed from their pot and the roots were washed with distilled water to remove as much soil as possible. Thereafter, three locations along the root were randomly selected at 2 cm intervals. Subsequently, 100 root tips for each location of the seeding roots were examined, and the number of mycorrhizal root tips (T) and non-mycorrhizal root tips (N) were counted under a dissection microscope (Olympus SZX7). The percent root colonization rate (CR) was calculated as follows [11]:

$$\mathrm{CR}\ (\%) = (T/T+N) \times 100$$

3. Results

3.1. Inoculation of seedlings and mycorrhizal synthesis

ECM formation in both host plants was observed approximately 2.5 months after inoculation with spore suspensions of *T. huidongense* and *T. himalayense*. *Tuber huidongense* colonized all five seedlings of *Q. acutissima* and all five seedlings of *Q. dentata*. *Tuber himalayense* similarly colonized all 10 seedlings of *Q. acutissima*; however, only eight of the ten *Q. dentata* seedlings were colonized.

3.2. Molecular analysis of ECM

All of the ITS sequences amplified using mycorrhizal root tip DNA were similar to those of *T. huidongense* and *T. himalayense* ITS sequences deposited in GenBank. Analysis of the constructed phylogenetic trees confirmed that the base sequence of ECM belonged to the same phylogenetic group as that from the DNA of the fruiting bodies (Figures 1 and 2).

3.3. Morphological and anatomical characterization of ECM

After 8 months of inoculation, the *T. huidongense* and *T. himalayense* ECM that formed in the two host plants was characterized. Common to both host plants, the ECM mantle of *T. huidongense* was irregularly interlocked and the mycelium was simple, curved, tortuous, septate, and exhibited some right-angle ramification (Figure 3). However, the diameter of the unramified ends and the length and diameter of the cystidia in the ECM were significantly different between the two host plants (independent t-test, $p < 0.05$; Table 1). The ECM mantle of *T. himalayense* in both host plants was generally brown, with localized dark areas, and an irregular interlocking pattern (Figure 4). The mycelium was simple, tortuous, and septate. There were significant

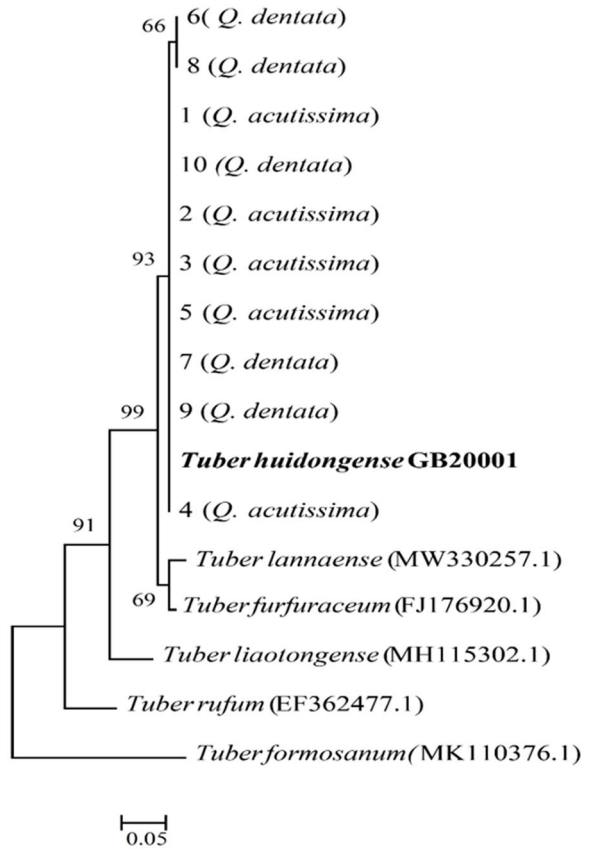

Figure 1. Neighbor-joining phylogenetic tree of *Tuber huidongense* GB20001 ascoma based on concatenated alignment of internal transcribed spacer (ITS) DNA sequences. *Tuber formosanum* was considered as an outgroup. The *T. huidongense* GB20001 sequence was isolated from the fruiting body. A total of 1–5 DNA sequences were derived from the ectomycorrhizae of *Quercus acutissima* and 6–10 DNA sequences were derived from the ectomycorrhizae of *Q. dentata*. Numbers in the figure represent bootstrap values (1000 replicates).

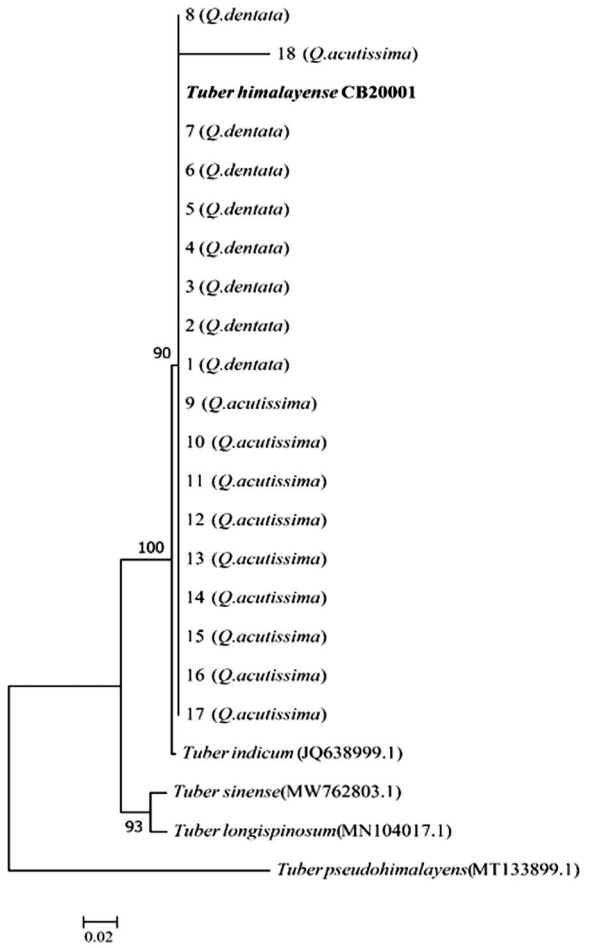

Figure 2. Neighbor-joining phylogenetic tree of *Tuber himalayense* CB20001 ascoma based on concatenated alignment of internal transcribed spacer (ITS) DNA sequences. *Tuber pseudohimalayense* was considered as an outgroup. The *T. himalayense* CB20001 sequence was isolated from the fruiting body. A total of 1–8 DNA sequences were derived from the ectomycorrhiza of *Quercus acutissima* and 9–18 DNA sequences were derived from the ectomycorrhizae of *Q. dentata*. Numbers in the figure represent bootstrap values (1000 replicates).

differences in the length of the ECM, length, and diameter of the unbranched end, thickness of the mantle, and length of the hyphae in the excised progenitor systems between the two host plants (independent *t*-test, $p < 0.05$; Table 2).

3.4. ECM root colonization rates

The mean CR of *T. huidongense* in *Q. dentata* was significantly higher than that in *Q. acutissima* ($p < 0.05$), whereas the opposite was observed in the case of *T. himalayense* (Figure 5). The mean CR of *T. himalayense* in *Q. acutissima* was significantly higher than that in *Q. dentata* ($p < 0.01$).

4. Discussion

In this study, mycorrhization was confirmed in both *Q. acutissima* and *Q. dentata* after 2.5 months of inoculation with spores of *T. huidongense* or *T. himalayense*. In previous studies, *T. huidongense* formed ECM with *Castanea mollissima* and *Pinus armandii* after approximately 5 months of inoculation [12], whereas *T. himalayense* formed ECM with *Quercus* spp. and *Pinus densiflora* species after 4 months of inoculation [13]. Thus, the time required for colonization may vary depending on the host plant species. Here, *Q. dentata* and *Q. acutissima* were shown to be the preferable host species for the cultivation of *T. huidongense* and *T. himalayense*, respectively.

Tuber spp. show a preference for specific host plant species [14]; therefore, selection of the appropriate host plant is an important factor for effective mycorrhization of *Tuber* spp. [2]. In this study, *T. huidongense* had a significantly higher CR in *Q. dentata* than *Q. acutissima*, and *T. himalayense* had a significantly higher CR in *Q. acutissima* than in *Q. dentata*. Thus, it was demonstrated that *T. huidongense* and *T. himalayense* exhibit a preference at the

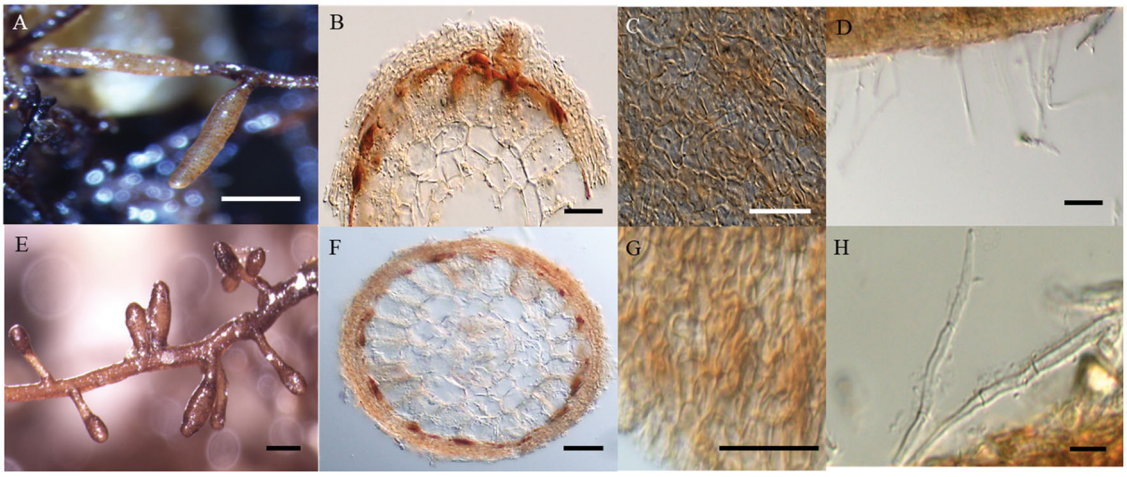

Figure 3. Macro-morphological and anatomical characteristics of *Tuber huidongense* mycorrhizae with *Quercus acutissima* (A–D) and *Q. dentata* (E–H). (A, E) Shape of mycorrhizal root tips. (B, F) Cross-section of mycorrhizal root tips. (C, G) Outer mantle surface structure. (D, H) Separate hyphae emanating from the outer mantle layer. (Scale bars: A, E = 1 mm; B, F = 20 μm; C, D, G, H = 50 μm).

Table 1. Dimensional measurements of *Tuber huidongense* ectomycorrhiza (ECM) in two *Quercus* plants.

Dimension	*Quercus acutissima*	*Quercus dentata*	*p*-Value	Iteration
ECM system (mm)	3.70 ± 0.25	4.28 ± 0.32	0.189	5
Length of unramified ends (mm)	2.16 ± 0.10	2.06 ± 0.06	0.367	30
Diameter of unramified ends (μm)	240.0 ± 4.05	203.3 ± 4.68	<0.001	30
Thickness of mantle (μm)	18.5 ± 1.20	15.17 ± 1.33	0.092	6
Length of cystidia (μm)	141.00 ± 4.94	194.00 ± 8.80	0.010	6
Diameter of cystidia (μm)	1.85 ± 0.07	2.10 ± 0.04	0.026	6

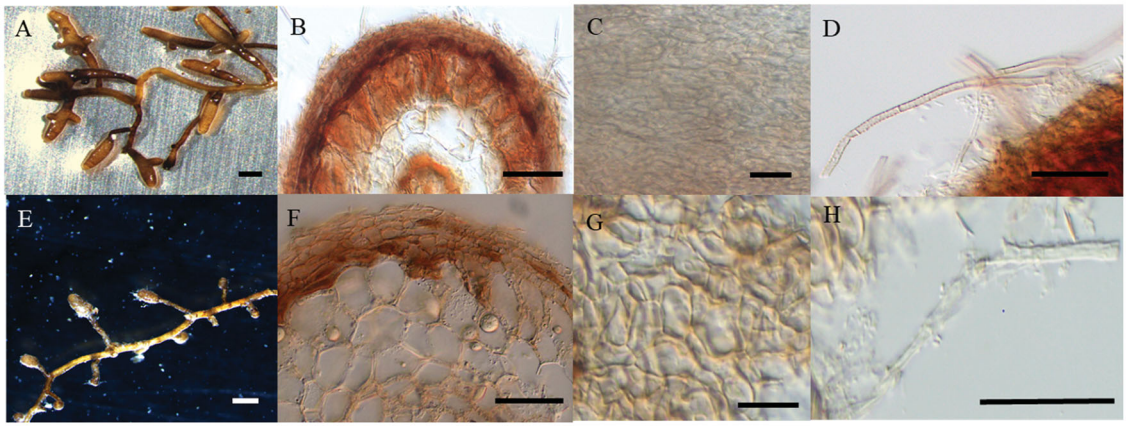

Figure 4. Macro-morphological and anatomical characteristics of *Tuber himalayense* mycorrhizae with *Quercus acutissima* (A–D) and *Q. dentata* (E–H). (A, E) Shape of mycorrhizal root tips. (B, F) Cross-section of mycorrhizal root tips. (C, G) Outer mantle surface structure. (D, H) Separate hyphae emanating from the outer mantle layer. (Scale bars: A, E = 1 mm; B, F = 30 μm; C, D, G, H = 25 μm).

Table 2. Dimensional measurements of *Tuber himalayense* ectomycorrhiza (ECM) in two *Quercus* species.

Dimension	*Quercus acutissima*	*Quercus dentata*	*p*-Value	Iteration
ECM system (mm)	5.34 ± 0.36	4.28 ± 0.17	0.189	5
Length of unramified ends (mm)	2.82 ± 0.07	1.75 ± 0.08	0.367	30
Diameter of unramified ends (μm)	208.70 ± 6.50	163.10 ± 7.68	<0.001	30
Thickness of mantle (μm)	15.33 ± 1.31	23.00 ± 1.86	0.092	6
Length of cystidia (μm)	146.57 ± 7.20	106.67 ± 6.03	0.010	6
Diameter of cystidia (μm)	2.54 ± 0.11	2.30 ± 0.06	0.026	6

level of the host plant species. Interestingly, fruiting bodies of *T. himalayense* have been generally reported in the rhizosphere of *Q. dentata* [9,15]; however, *T. himalayense* showed a higher CR in *Q. acutissima* than *Q. dentata* in the current study. Initial CR of *T. himalayense* could be higher in *Q. dentata* seedling than in *Q. acutissima* [13]; however, further studies are needed to determine whether initial CR affects the formation of the fruiting body.

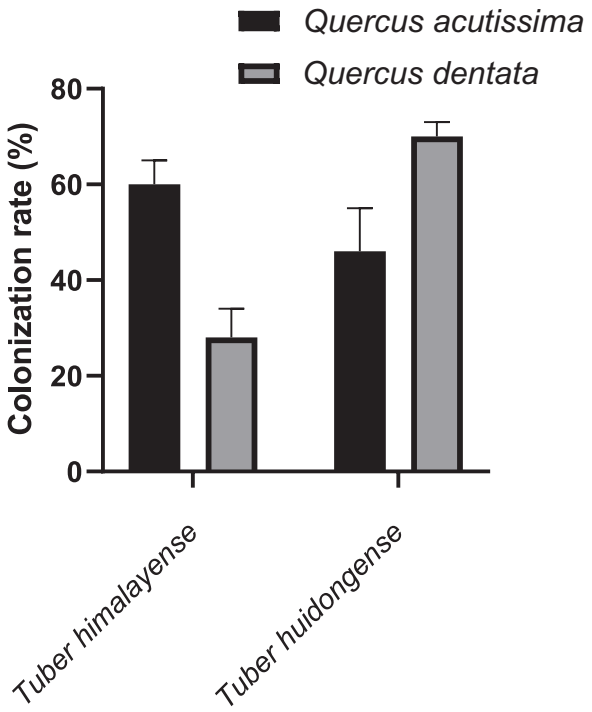

Figure 5. Ectomycorrhizal colonization rates of two oak trees (*Quercus acutissima* and *Q. dentata*) inoculated with two truffle species (*Tuber himalayense* and *T. huidongense*) collected in Korea. Values represent the mean ± standard deviation. $^{*}p < 0.05$; $^{**}p < 0.01$.

The morphological and anatomical characteristics of *T. huidongense* and *T. himalayense* ECM were described in this study, representing the first characterization of *T. huidongense* ECM formed on oak trees. Morphological differences in *T. huidongense* ECM between the host plants *Castanea mollissima* and *Pinus armandii* were previously reported [12], further supporting the conclusion that ECM can show distinct morphological differences according to the host plants, even when inoculating with the same *Tuber* species. Most of the *T. huidongense* ECM that formed in *C. mollissima* and *P. armandii* exhibited only bright colors, whereas the ECM that formed in oak trees was dark in color [12]. However, it is difficult to conclude that this result is due to the host plant species; the color of ECM changes with age and it can be affected by the specific conditions applied to the growing seedlings [16].

The current study also characterized the properties of *T. himalayense* ECM formed on *Q. dentata* for the first time. There have been previous reports of the properties of *T. himalayense* ECM formed on *Q. acutissima*, *Quercus serrata*, *Quercus phillyraeoides*, and *P. densiflora* [13]. Most of the ECM characteristics reported for *T. himalayense* in these host plants were similar to those in *Q. dentata*; however, we showed that the ECM mantle in *Q. dentata* differed from the polygonal or angular-shaped mantle cells in *P. densiflora* [13]. In this study, slight differences in ECM morphology were observed depending on each *Tuber* spp., even if the ECM was formed in the same host plant. For example, when the ECM characteristics of *T. huidongense* and *T. himalayense* were compared 8 months after inoculation, brown spots were observed in some ECM in *T. huidongense*, and some of the mycelia had right-angle ramifications. In addition, the mantel color of *T. himalayense* ECM was darker than that of *T. huidongense*.

By observing the ECM of *T. huidongense* and *T. himalayense*, it was possible to understand their characteristics according to *Tuber* species, and their fluctuations according to host plant species. However, it is difficult to distinguish *Tuber* spp. through ECM observations alone. Therefore, additional studies on *T. huidongense* and *T. himalayense* ECM are required. This study expanded the host plant range of *T. huidongense* and *T. himalayense* by demonstrating the formation of ECM in *Q. acutissima*, in addition to *Q. dentata*. For these results to lead to new agricultural activities, additional follow-up experiments are needed to determine the selective conditions for ECM synthesis of *T. huidongense* and *T. himalayense*.

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ORCID

Ahn-Heum Eom http://orcid.org/0000-0002-6821-1088

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