Efficient establishment of pure cultures of yellow chanterelle *Cantharellus anzutake* from ectomycorrhizal root tips, and morphological characteristics of ectomycorrhizae and cultured mycelium

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ABSTRACT

Species of fleshy yellow *Cantharellus* are known as chanterelles, which are among the most popular wild edible mycorrhizal mushrooms in the world. However, pure culture isolates of *Cantharellus* are rare. We report an efficient isolation technique of the Japanese golden chanterelle, *Cantharellus anzutake*, from its ectomycorrhizal root tips. Field-sampled fresh ectomycorrhizal root tips of *C. anzutake* on various hosts such as pines, spruce, and oaks were vortexed with 0.005% Tween 80 solution, surface sterilized with 1% calcium hypochlorite solution, rinsed with sterilized distilled water, and placed on modified Norkrans' C (MNC) agar plate medium. Most ectomycorrhizal root tips of *C. anzutake* produced yellowish mycelial colonies within a few months. In contrast, tissue isolation from basidiomata provided limited cultures of *C. anzutake* but much contamination of bacteria and molds, even on media that contained antibiotics. The established *C. anzutake* cultures had clamp connections on the hyphae and contained intracellular oily droplets. These cultured isolates were identified as *C. anzutake* by sequence analysis of the rRNA internal transcribed spacer (ITS) region and translation elongation factor EF1-alpha (*tef-1*) genes.

Keywords

Carotenoid, Colony growth, Edible mushrooms, Mycorrhizal anatomy, Mycorrhizal isolation

1. Introduction

Yellow chanterelles have attracted much attention from people who enjoy these attractive mushrooms as a delicacy. The market value of chanterelles is estimated at more than 1 billion dollars annually, which is the maximum sum of all wild edible mushrooms sale worldwide (Watling, 1997; Hall, Wang, & Amicucci, 2003; Pilz, Norvell, Danell, & Molina, 2003). The representative *Cantharellus cibarius* Fr., the European golden chanterelle, is endemic to Europe and probably other boreal regions of the Eurasian continent (Buyck, Kauff, Eyssartier, Couloux, & Hofstetter, 2014; Olariaga et al., 2017; Ogawa, Endo, Fukuda, & Yamada, 2018). Other commercially valued species include *C. pallens* Pilát and *C. ferruginascens* P.D. Orton in Europe and *C. formosus* Corner and *C. roseocanus* (Redhead, Norvell & Danell) Redhead, Norvell, & Moncalvo in North America (Pilz, Norvell, Danell, & Molina, 2003; Buyck, 2016). Study of the cultivation of these mushrooms is relatively challenging, and a very limited number of trials have been reported due to the difficulty of manipulating this mushroom group in vitro and in situ (Danell, 1994; Danell & Camacho, 1997; Pilz, Norvell, Danell, & Molina, 2003).

It is generally accepted that it is difficult to establish pure cultures from basidiomata tissues of *C. cibarius* due to considerable contamination by bacteria and fungi (Modess, 1941; Schouten & Waandrager, 1979; Danell & Fries, 1990; Danell, 1994). However, Fries (1979) reported validly characterized pure cultures of *C. cibarius*. Strain 740b established by multi-spore isolation on nutrient agar medium showed a yellowish colony color and a specific fragrance similar to that of the fruit body of *C. cibarius* (Fries, 1979; Straatsma, Konings, & van Griensven, 1985). This strain was subsequently identified as a true *C. cibarius* culture by a DNA hybridization technique (Straatsma, Konings, & van Griensven, 1985). Straatsma, Konings, & van Griensven (1985) reported that 70 tested fruit bodies of *C. cibarius* provided

20 cultures by tissue isolation and that 40 fruit bodies provided 13 cultures by spore isolation, most of which showed similar characteristics to strain 740b. Among the 20 isolates from basidiomata tissue, 3 were obtained on half-strength and malt-deleted Fries medium (Fries, 1978), and 15 were obtained on Murashige and Skoog medium (Murashige & Skoog, 1962) as modified by Meredith (1979). Danell & Fries (1990) reported 56 cultured strains of *C. cibarius* and four of *C. pallens* that were isolated from tissues of fruit bodies collected in coniferous forests. Based on established cultures, in vitro ectomycorrhization of *C. cibarius* was first presented by Moore, Jansen, & Vangriensven (1989). Danell (1994) reported a refined system for establishing large pine ectomycorrhizal seedlings associated with *C. cibarius* in vitro. This system progressed to produce fruit bodies of *C. cibarius* in association with host pine seedlings in pots under greenhouse conditions (Danell & Camacho, 1997; Pilz, Norvell, Danell, & Molina, 2003). However, whether fungal isolation of *C. cibarius* from ectomycorrhizal samples is feasible has yet to be reported.

We recently described a fleshy yellow chanterelle, *C. anzutake* W. Ogawa, N. Endo, M. Fukuda & A. Yamada, which was identified previously as *C. cibarius* in Japan (Ogawa, Endo, Fukuda, & Yamada, 2018). This species is quite similar to the European *C. cibarius* in morphology and phylogenetic position. Therefore, the Japanese yellow chanterelle has significant potential as a gourmet food. However, it was inferred that fungal isolation of this species from basidioma tissues would be difficult due to probable endogenous microorganisms, as is the case for the European *C. cibarius*. Given this circumstance, fungal isolation from ectomycorrhizal root tips could be an alternative approach for establishing cultures of this ectomycorrhizal species (Yamada, Ogura, Degawa, & Ohmasa, 2001, Endo et al., 2015). This technique is generally applicable to diverse fungal taxa (Yamada & Katsuya, 1995) and throughout the year, irrespective of the mushroom fruiting season, as long as fresh ectomycorrhizal samples are obtained (Yamada, Ogura, Degawa, & Ohmasa, 2001; Endo et

al., 2015). Once we had marked the precise fruiting points of chanterelle in a forest site, we were able to analyze the ectomycorrhizae present in the soil at that location in post-fruiting seasons. Ectomycorrhizae of chanterelles have been described for *C. cibarius* (Mleczko, 2002a, 2004a) and *Craterellus tubaeformis* (Fr.) Quél. (Mleczko, 2002b, 2004b; Fransson, 2004), suggesting common characteristics among these ectomycorrhizae such as oily droplets in the mantle mycelium, which should facilitate their routine sampling. Therefore, in this study, we aimed to test the establishment of pure cultures of the Japanese yellow chanterelle from ectomycorrhizal root tips and from basidioma tissues and to compare the efficiency of both isolation techniques.

2. Materials and methods

2.1. Basidiomata and ectomycorrhizal samples

We sampled basidiomata of *C. anzutake* from various forest sites (Table 1). After preparation for fungal isolation, as described below, fresh basidiomata samples were freeze-dried and stored as dried specimens in the mycorrhiza laboratory of the Faculty of Agriculture, Shinshu University (Ogawa, Endo, Fukuda, & Yamada, 2018).

Ectomycorrhizal samples were collected as soil samples from beneath basidiomata of *C. anzutake* (Table 1) when the basidiomata were collected or at a later time. A soil sample of about 100–200 cm^3 was collected from beneath each basidioma (Table 1). Collected soil samples were stored at 5 °C in a refrigerator until processing (for several days at most after sampling). Each of the collected soil samples was placed on a metal sieve (mesh size: 1 mm), and mycorrhizal root systems were rinsed under tap water. The washed root systems having probable chanterelle ectomycorrhizal root tips were transferred to a Petri dish filled with distilled water. The ectomycorrhizal root tips of *C. anzutake* showed a yellowish or cream-

yellow fungal mantle under a stereomicroscope (Stemi 2000C, Carl Zeiss AG, Jena, Germany). These ectomycorrhizal root tips were excised and placed into another Petri dish filled with distilled water, and small soil particles and organic debris were detached from the fungal mantle surface using a fine paintbrush and fine forceps. To confirm whether the cleaned ectomycorrhizal root tips were chanterelle-associated, a few chanterelle-like ectomycorrhizal root tips from each washed ectomycorrhizal sample were prepared on a glass slide and mounted with lactic acid, observed under a differential interference contrast (DIC) microscope (AXIO Imager A1, Carl Zeiss AG), and photographed (D90, Nikon Imaging Japan, Inc., Tokyo). The combination of hyphal clamp connections and intrahyphal oily droplets distinguishes chanterelle ectomycorrhizae from those of other taxa. At least six ectomycorrhizal root tips from each soil sample were transferred to an autoclaved 1.5-mL microtube and processed for fungal isolation.

2.2. Fungal isolation from basidioma tissue

Sampled fresh basidiomata were tested for isolation within a few days. The basidioma surface was wiped with cotton gauze moistened with 70% ethanol, and the pileus was cut into two parts on a clean bench using a sterilized scalpel. Several basidiomata tissue samples, each ca. 30–50 mm^3 and cubic in shape, were cut from the flesh of the pileus and inoculated onto MNC agar medium (Yamada & Katsuya, 1995) containing different antibiotics or combinations of antibiotics, i.e., 100 mg/L streptomycin and 50 mg/L tetracycline, 100–200 mg/L penicillin, or 100 mg/L chloramphenicol. The inoculated plates were incubated in the dark at 20 °C. If the targeted mycelium grew on the agar surface, its margin was transferred onto another fresh MNC agar plate that did not contain any antibiotics.

2.3. Fungal isolation from ectomycorrhizal root tips

The fungal isolation procedure followed the method of Endo et al. (2015), with minor modification. Selected ectomycorrhizal root tips previously placed in a 1.5-mL microtube, as described above, were soaked in 1 mL of sterilized 0.01% Tween 80 solution and vortexed for 1 min. The washed solution was removed by pipetting and replaced with another fresh 0.01% Tween 80 solution. This washing procedure was replicated three times. The vortexed ectomycorrhizal root tips were transferred using fine forceps into a 75-mL glass bottle filled with 50 mL of autoclaved 0.005% Tween 80 solution and the sample was agitated using a magnetic stirrer for 30 min. The fully washed ectomycorrhizal root tips were then transferred using fine forceps into a 75-mL glass bottle containing 50 mL of 1% $Ca(ClO)_2$ for surface sterilization during 1–2 min. Ectomycorrhizal samples were then poured onto a metal sieve (mesh size: 200 μm) that was set on a glass beaker, and then quickly transferred using fine forceps into a 75-mL glass bottle containing 50 mL of sterilized distilled water. The root tips were rinsed for 1 min, and this rinsing procedure was replicated three times with fresh sterilized, distilled water. The ectomycorrhizal root tips were finally transferred onto a Petri dish, and each tip was sectioned into several small segments using a sterilized razor. Five to ten ectomycorrhizal segments were inoculated onto an MNC agar plate or a 0.1× strength MNC agar plate, both of which contained 100 mg/L streptomycin and 50 mg/L tetracycline. The inoculated agar plates were incubated in the dark at 20 °C. If the expected mycelial colonies, i.e., those with slow growth, yellowish to cream-yellow color, and non-sporulating mycelium, grew on the agar surface, the mycelium was subcultured onto another fresh MNC agar plate or 0.1× strength MNC agar plate that did not contain any antibiotics. If the subcultured mycelium grew and established a new colony characteristic of *C. anzutake*, we determined it to be a successful isolation and calculated the fungal isolation ratio. Colonies of other mycelial fungi, yeasts, and bacteria were discarded.

2.4. Molecular identification of established chanterelle culture strains

The culture strains established from basidiomata and ectomycorrhizae were analyzed for their internal transcribed spacer (ITS) region of the rDNA by restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) amplicons. DNA extraction and PCR amplification procedures were conducted according to Ogawa, Endo, Fukuda, & Yamada (2018). PCR amplicons were digested with *Hin*f I, *Hae* III, and *Rsa* I according to the manufacturer's recommendations. If necessary, DNA sequencing of the rDNA ITS2 region or translation elongation factor EF1-alpha (*tef-1*) gene (Ogawa, Endo, Fukuda, & Yamada, 2018) was conducted. Some of the established *C. anzutake* culture strains were deposited in the culture collection at the NITE Biological Resource Center (NBRC), Japan.

2.5. Microscopic characterization of established chanterelle culture strains

The cultured *C. anzutake* strains on MNC medium were observed for their hyphal characteristics by DIC and fluorescent microscopy (Axioplan 2 Imaging, Carl Zeiss AG) with a 100× oil immersion lens (Plan NEOFLUAR) and Filter set 01 (488001-9901-000: excitation, BP 365/12; beam splitter, FT 395; emission, LP 397) and photographed (D200, Nikon Imaging Japan, Inc.). The karyotic status of the hyphae was observed by staining with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Japan, Tokyo). Mycelium grown in MNC medium for 1 mo was soaked in distilled water, and a small amount of mycelium was mounted with 10 µL of 1 mg/L DAPI solution on a glass slide. The margin of the cover glass was sealed with nail coat to prevent evaporation of the DAPI solution. After 10–20 min of staining, the mycelium was irradiated with UV radiation, and the visible spectrum excited was observed. To observe the presence/absence of other intracellular structures such as

polyphosphate, mycelium was mounted with 10 mg/L DAPI solution. In addition, hyphae were observed for additional intracellular characteristics such as vacuoles or oily droplets and fine granules in the secretory hyphae by mounting them with distilled water or lactic acid. Intracellular fine structures observed by DIC microscopy were highlighted by the focus stacking and deconvolution functions of Adobe Photoshop CC (Adobe System, Inc., San Jose, CA). For this process, 2–3 differently focused photographs (depth: ca. 1 μm) were taken of the same hyphae. To reveal the precise positions of fluorescent signals on the hyphae, a fluorescent micrograph was overlaid on the DIC micrograph using Adobe Photoshop CC.

3. Results

3.1. Sampling of chanterelle-associated ectomycorrhizal root tips

We tested 17 basidioma samples and 18 ectomycorrhiza samples of *C. anzutake* for the fungal isolation (Table 1). Ectomycorrhizal root tips of *C. anzutake* showed yellowish to light-brownish color and were often accompanied by whitish, cottony extraradical mycelium (Fig. 1A, B). The fungal mantle thickness (ca. 20–30 μm) was mostly the same at the apex and the base of an ectomycorrhizal root tip, but was sometimes thicker at the base. The outer layer of the fungal mantle showed plectenchymatous type-B ramification (Agerer, 1990) of mycelium (Fig. 1C), and the inner layer had plectenchymatous type-H and pseudoparenchymatous type-M ramifications (Fig. 1D). These fungal mantle mycelia often contained intracellular yellowish or transparent oily droplets. In addition, strong yellow or orange-yellow crystals were observed intra- or intercellularly in some fungal mantle hyphae (Fig. 1E).

3.2. Fungal isolation from basidiomata

Cantharellus anzutake was isolated from 3 of the 17 tested basidioma samples

(Table 2). Two isolates were obtained on MNC agar medium with streptomycin and tetracycline, and one was obtained on MNC agar medium with chloramphenicol. Most of the inocula, however, showed contaminated microbial growth, i.e., sporulating mycelial fungi, yeast cells, and bacteria. No cultures were obtained under other nutrient medium conditions.

3.3. Fungal isolation from ectomycorrhizal root tips

Cantharellus anzutake was isolated from 15 of the 18 tested ectomycorrhizal samples (Table 3). Although the fungal isolation ratio was different for each ectomycorrhizal sample and ectomycorrhizal root tip, some ectomycorrhizal samples provided *C. anzutake* mycelium from most of the tested ectomycorrhizal root tips and sectioned segments. At the generic level of the host plants, the averaged isolation ratio was 12–49%. In relation to season, two unsuccessful samples were tested in Aug, and another three samples that showed a hyphal recovery ratio of less than 10% were tested in Jun–Aug. However, the highest isolation ratio occurred for sample C-23-M tested in Aug. Contamination of microorganisms was not as common as in the case of isolation from basidiomata tissue. In relation to the isolation seasons, both summer and winter samples recovered *C. anzutake* cultures without any distinct difference in isolation ratio.

The isolation ratio was also compared among the six paired basidomata and ectomycorrhizae samples (Supplementary Table 1). Isolation ratios were significantly higher in ectomycorrhizal samples (36.8%) than basidioma samples (1.7%).

3.4. Mycelial characteristics of C. anzutake on MNC medium

The established pure culture strains of *C. anzutake* showed yellowish colonies (Fig. 2A–C, E, F). They showed a mycelial growth rate of around 20 mm/mo on MNC agar at 20 °C and better mycelial growth on MNC or 10 × diluted MNC than on modified Melin

Norkrans (MMN) medium (Marx, 1969), malt agar, or potato dextrose agar media (data not shown). Young and growing colonies presented an apricot-like smell on MNC, as is the case for fresh basidiomata of this species and for *C. cibarius* cultures (Straatsma, Konings, & van Griensven, 1985). Hyphae at the colony margin were 2–4 μm diam, and those inside the colony were 2–5 μm diam, with a clamp connection on the septa (Fig. 2D–K). Dikaryotic status was observed on the clamped hyphae (Fig. 2G, H) showing dolipore septa (Fig. 2I). Some matured, and thick-walled hyphae, a probable storage region of exploiting hyphae or secretory hyphae (Clemençon & Emmett, 2004), showed strong to moderate whitish–whitish-blue autofluorescence with UV irradiation (Fig. 2J), which corresponded to intracellular crystal-like structures or fine granular material (Fig. 2H). In contrast, intracellular oily droplets, most of which were larger than 0.5 μm diam (Fig. 2D, K), did not show autofluorescence with UV irradiation.

PCR-RFLP analysis of those cultured mycelia in each established strain showed the identity of cultures and the related basidiomata specimens (Fig. 3). Some strains were sequenced at the ITS and *tef-1* regions and deposited at the DDBJ site (https://www.ddbj.nig.ac.jp/index-e.html) (Tables 2, 3).

4. Discussion

In this study, we tested ectomycorrhizal root samples of chanterelle for fungal isolation to pure culture establishment for the first time, and obtained more successful data than with basidioma tissue isolation. Pure cultures of *C. anzutake* were established from more than 80% of the ectomycorrhiza samples tested on MNC agar medium (Table 3). Even in the unsuccessful ectomycorrhiza sample C-19-M, mycelial growth of *C. anzutake* occurred on the

inoculated ectomycorrhizal segments but did not propagate on MNC agar medium (data not shown). By contrast, tissue isolation showed limited isolation success (Tables 2, Supplementary Table 1). Therefore, fungal isolation from ectomycorrhizal root tips was quite successful for *C. anzutake*. In addition, 11 ectomycorrhizal samples were not isolated from their related basidomata because they were fully matured or injured, decreasing their isolation value (Table 1). Therefore, isolation ratios among the basidomata samples would likely be much lower than those of ectomycorrhizal isolation samples if non-tested basidiomata had been tested. Straatsma, Konings, & van Griensven (1985) established 20 cultures from 70 basidiomata of *C. cibarius* tested on Fries medium, and Danell & Fries (1990) established 56 cultures from 229 basidiomata of *C. cibarius* tested on this medium. Therefore, our isolation ratio of *C. anzutake* from ectomycorrhizal root tips can be recognized as particularly successful. To verify the efficiency of our study, we should try this ectomycorrhizal isolation technique with samples of European *C. cibarius* populations on MNC and Fries media. All cultured *C. anzutake* strains showed intracellular oily droplets with some variation of their frequency (Fig. 2). Therefore, those intracellular oily droplets are a common feature of *C. anzutake* and probably of other yellow chanterelles. In this study, we did not clarify the relationship between yellowish tone of colony color and quantity of intracellular oily droplets because we mainly conducted microscopic characterization of young mycelium that showed whitish color (Fig. 2A).

The isolation ratio of *C. anzutake* from ectomycorrhizal root tips on MNC agar medium showed a similar trend to the cases of *Tricholoma matsutake* (S. Ito & S. Imai) Singer and *T. bakamatsutake* Hongo (Yamada, Ogura, Degawa, & Ohmasa, 2001; Endo et al., 2015). Yamada, Ogura, Degawa, & Ohmasa (2001) suggested that the fungal isolation technique from ectomycorrhizal root tips is applicable throughout the year, irrespective of the fruiting season of basidiomata, and that the isolation ratio of ectomycorrhizal samples can be

compared seasonally among the fungal species; this was also the case for *C. anzutake* (Fig. 2; Table 3). We observed that ectomycorrhiza samples processed in winter–spring were more likely to show successful mycelium isolation ($P < 0.01$ by the t-test; Tables 1, 3). On the other hand, samples processed in summer showed large variation in the isolation ratio, which might have been caused by sample degradation at higher seasonal temperatures. We tested only segmented ectomycorrhizal root tips for fungal isolation in the present study. Future studies should also test non-segmented, intact ectomycorrhizal root tips to determine how such an ectomycorrhizal preparation procedure might affect the fungal isolation ratio, and whether distinct seasonal variation in isolation success is related to fungal phenological activity.

Ectomycorrhizal root tips of *C. anzutake* showed a characteristic yellowish and cottony fungal mantle, as well as many intrahyphal oily droplets and strong yellow to orange-yellow crystals (Fig. 1). These hyphal characteristics were common with those of the pileus mycelium of this fungal basidiomata (Ogawa, Endo, Fukuda, & Yamada, 2018). Therefore, ectomycorrhizal root system colonized by *C. anzutake* was easily detectable beneath its basidiomata. The intrahyphal oily droplets and strong-yellow to orange-yellow crystals are probable carotenoid lipids, such as carotenes and xanthophylls (Haxo, 1950; Danell, 1999; Pilz, Norvell, Danell, & Molina, 2003; Reczyński et al., 2013). Basidioma tissue of yellow chanterelles contains a relatively large amount of bicyclic carotenoids compared to many other species in the fungal kingdom (Gill & Steglich, 1987; Danell, 1999; Pilz, Norvell, Danell, & Molina, 2003; Velíšek & Cejpek, 2011; Reczyński et al., 2013). If these compounds are simply identified in ectomycorrhizal samples under a microscope with the aid of specific chemical reactions, the mycorrhizal preparation step can be shortened, and the targeted chanterelle samples can be selected validly. Most of our established culture strains showed yellowish colony color and contained many intracellular oily droplets (Fig. 2). As whole genome sequences of the cultured strain C-23 has been released on the JGI Genome

Portal (https://genome.jgi.doe.gov; Aug 21, 2017), such chanterelle-specific chemical compounds can serve as targets for research on both fungal taxonomy and cultivation based on metabolic pathway.

The present data also provide new insight into chanterelle–microbial interactions and their ecophysiological significance. *Cantharellus cibarius* has been reported to have quite diverse interactions with bacteria and mold in their basidiomata tissue, which is atypical in basidiomycetous mushroom fungi (Straatsma, Konings, & van Griensven, 1985; Danell, 1994, 1999; Pilz, Norvell, Danell, & Molina, 2003). Although inner pileal tissue of *C. anzutake* basidiomata also contained much bacteria and mold, as was the case for *C. cibarius*, its ectomycorrhizal fungal mantle and more inner tissue areas, i.e., Hartig net area and root vascular system, contained very limited microbes. This suggests that the abundant microbes in the chanterelle basidiomata represent a periodically specific event in the life cycle of chanterelles. Danell (1999) briefly reported that chanterelle-associated bacteria obtained from basidiomata did not act as mycorrhization helper bacteria. These results reinforce the hypothesis that those chanterelle-associated microbes occur specifically during basidioma morphogenesis and could be efficiently dispersed by mycophagic organisms of the host chanterelles. Microbes colonizing *C. cibarius* basidioma tissue can grow using intercellular exudates from the host chanterelle hyphae (Rangel-Castro, Danell, & Pfeff, 2002a; Rangel-Castro, Danell, & Taylor, 2002b). Therefore, yellow chanterelle–microbe interactions at the reproductive phase of chanterelles would have evolutionary significance for the harbored microbes as probable commensalism. By contrast, the evolutionary significance is not clear for yellow chanterelles, although those microbes may be involved in the promotion of chanterelle reproduction, such as by influencing spore germination.

In conclusion, we established pure cultures of yellow chanterelles from ectomycorrhizal root tips at a high success rate using the mycorrhizal isolation technique.

This approach will be applicable to other chanterelle species, including *C. cibarius*, and is therefore valuable for cultivation studies of yellow chanterelles.

Disclosure

The authors declare no conflicts of interest. All of the experiments in this study were performed in compliance with the current laws of Japan.

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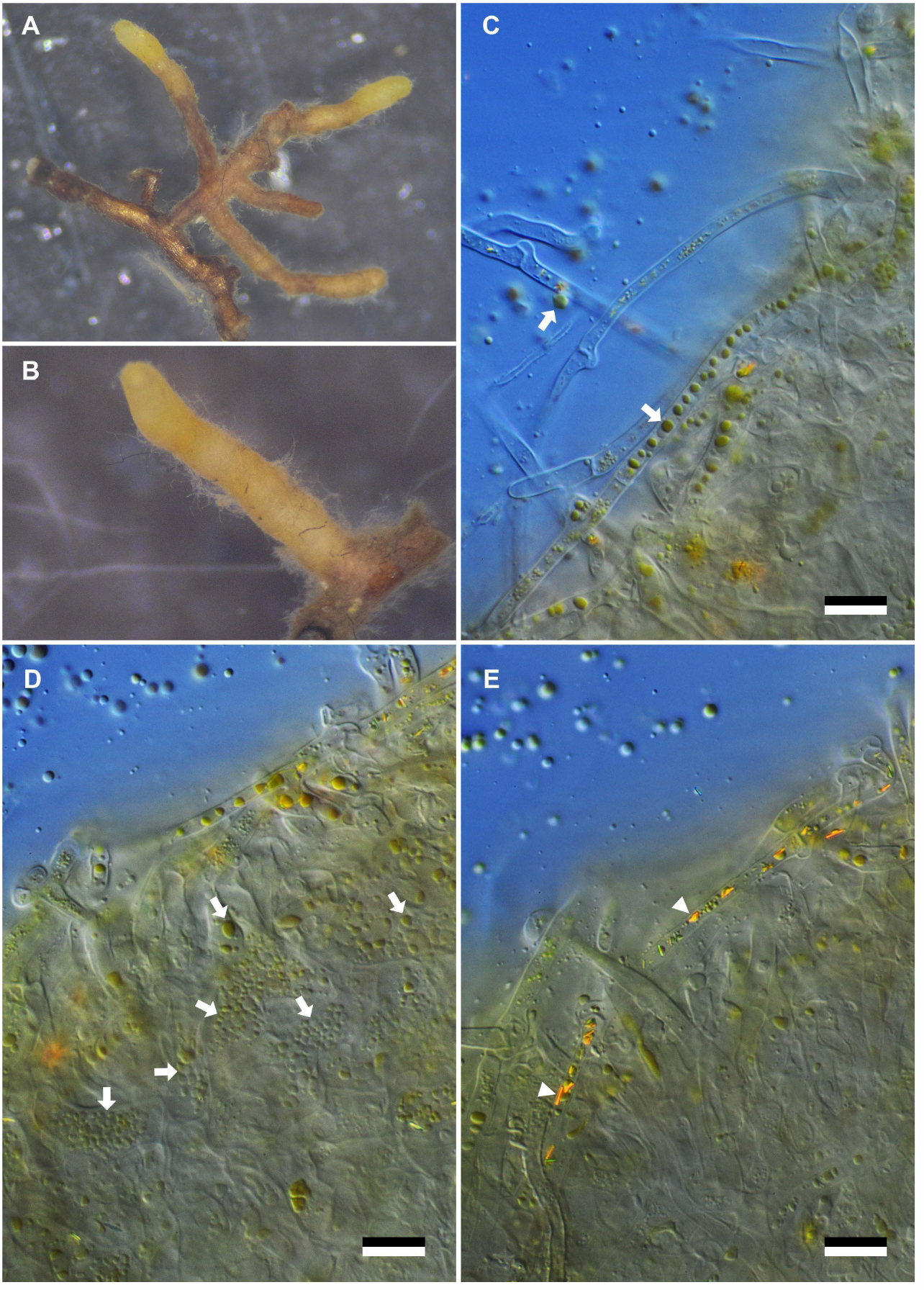
Figure legends

Fig. 1. Morphological characteristics of ectomycorrhizal root sample S-235-M. A: External morphology of the ectomycorrhizal root system showing the ocher color of the root tips. B: A magnified ectomycorrhizal root tip showing distinct extraradical cottony mycelium. C: Extraradical hyphae and surface mantle hyphae having a clamp connection at the septum and intracellular yellowish oily droplets (arrows). D: Inner mantle mycelium having pseudoparenchymatous structure and also containing intracellular oily droplets (arrows). E:

Bright orange-yellow colored probable extrahyphal crystals on surface mantle hyphae (arrowheads). These intra- and interhyphal characteristics (C–E) are common with those of pileus mycelium of *C. anzutake* basidiomata (Ogawa et al., 2018). Granules floating in the mounted lactic acids (C–E) are leaked oily droplets from the ectomycorrhizal mantle mycelium. *Bars*: C–E 10 μm.

Fig. 2. Morphological characteristics of cultured mycelium of *Cantharellus anzutake* strains C-18 (A–D), EN-60 (E), EN-61 (F), S-235 (G, H) and S-236 (I–K) on MNC agar plate (A–F) or liquid media (G–K). A: Growing colony mycelia. B: A young colony that had just started mycelial growth on the agar surface (whitish in color) from the inoculated mycelium with agar block (yellowish in color), which is the magnified figure of the right-side colony of Fig. 2A. C: Matured colonies. D: Hyphae of the colony margin on agar, which show clamp connections and intracellular oily droplets. Mycelium was mounted with lactic acid. E, F: Matured colonies on agar showing color difference between strains. G: DAPI-stained (1 mg/L) paired nuclei at the center of a hyphal cell that are delimited on both side by clamped septa. H: DAPI-stained (10 mg/L) paired nuclei (blue fluorescence) surrounded by probable polyphosphate fine granules (yellowish green fluorescence), ended by the backside vacuoles (no fluorescence). Such hyphae were often thick-walled. I: A hypha showing a clamped septum with dolipore at the center and several vacuoles. Mycelium was mounted with distilled water. J: Autofluorescence by UV-irradiated matured hyphae (white to whitish-blue in color) where crystal-like material or fine-granular material had filled. Some of these hyphae are thick-walled. Mycelium was mounted with lactic acid. K: Intracellular oily droplets that were negative to UV irradiation. *Bars*: D, G–K 10 μm.

Fig. 3. Restriction fragment length polymorphism (RFLP) patterns of cultured C-18 strain and its related basidioma specimen C-18. Lane M: molecular marker (0.1–3.0 Kbp). Each B (basidioma specimen) and C (cultured mycelium) lane pair exhibited equal band sizes in intact polymerase chain reaction (PCR) amplicons and enzyme digestions (*Hin*fI, *Hae*III, and *Rsa*I).


A
B
C
D
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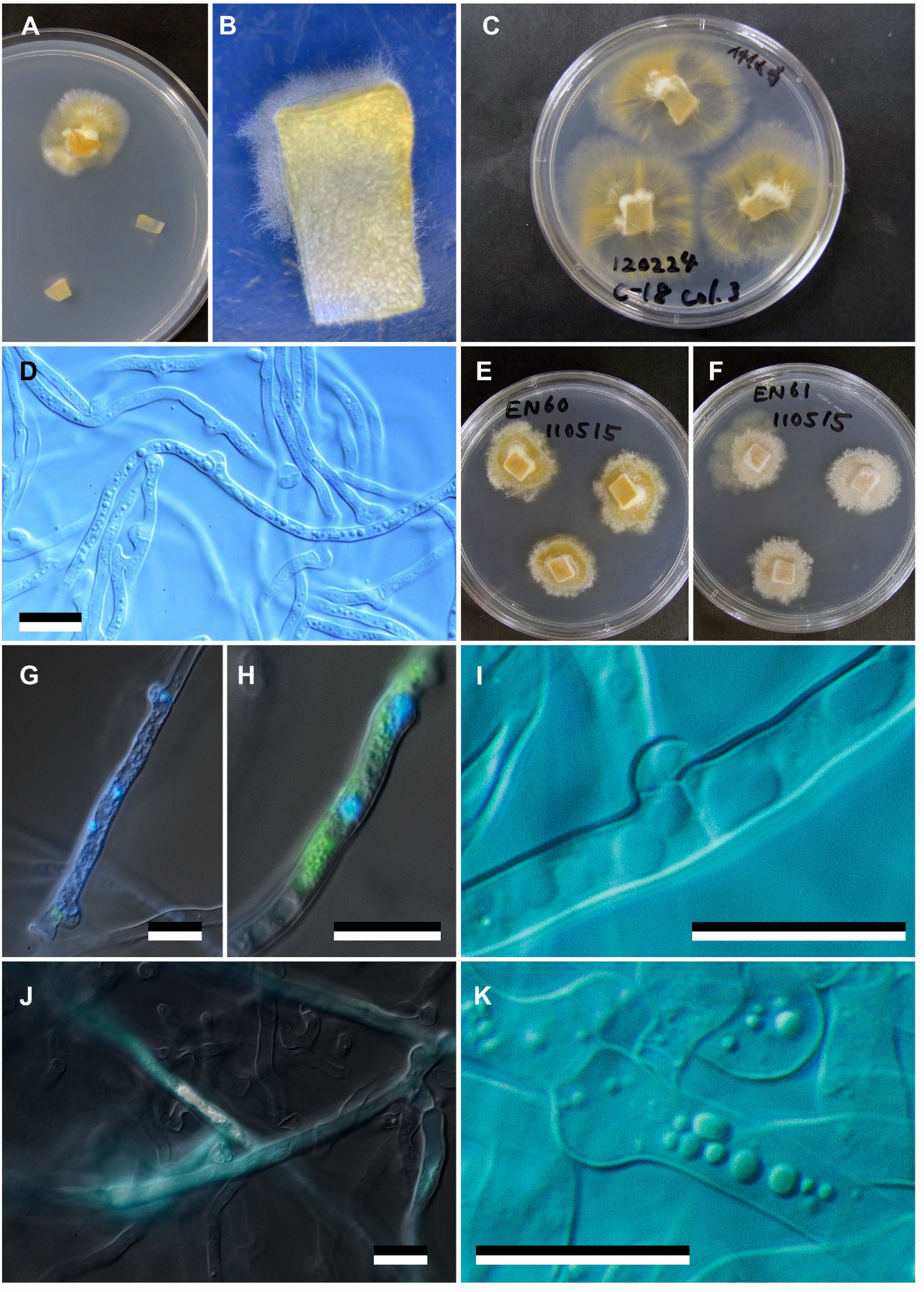
A
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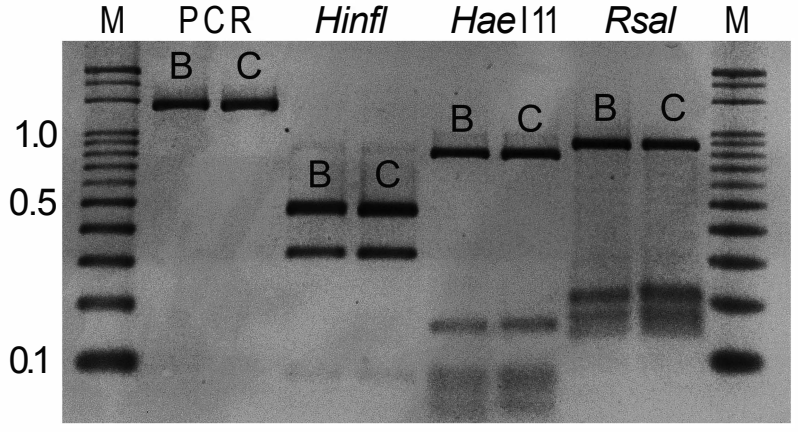
M
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Hinfl
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B
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B
C
1.0
0.5
0.1

Table 1 Basidiomata and ectomycorrhizae of *Cantharellus anzutake* tested for isolation.

Sample ID		Date of sampling	Sampling site	Forest canopy species[a]	Ecotomycorrhizal host[a]	Related basidioma sample	
Basidioma specimen	Ectomycorrhizae					ID	Reference
	C-30-M	28 Aug 2011	Shichiga-hama-machi, Miyagi	Pd, Pt	Pt	S-117[b]	Ogawa et al. 2018.
	S-210-M	27 Jun 2011	Nagano-shi, Nagano	Pd	Pd	8508[b]	
	C-23-M	5 Aug 2011	Matsumoto-shi, Nagano	Qc, Bp	Qc	C-23[b]	Ogawa et al. 2018.
	C-25-M	7 Aug 2011	Sakuho-machi, Nagano	Qc	Qc	ND[b]	
	C-26-M	7 Aug 2011	Sakuho-machi, Nagano	Qc, Bp, Pd	Qc	C-26[b]	Ogawa et al. 2018.
	C-12-M	3 Aug 2011	Minowa-machi, Nagano	Pd	Pd	C-12[b]	Ogawa et al. 2018.
	C-18-M	5 Aug 2011	Ina-shi, Nagano	Pd	Pd	C-18[b]	Ogawa et al. 2018.
	C-19-M	5 Aug 2011	Ina-shi, Nagano	Pd	Pd	C-19[b]	Ogawa et al. 2018.
S-25		25 Jul 2009	Minami-minowa-mura, Nagano	Pa			
S-89		18 Jul 2010	Minami-minowa-mura, Nagano	Pa			Ogawa et al. 2018.
	S-201-M	17 Dec 2010	Minami-minowa-mura, Nagano	Pa	Pa	S-25, S-89	
	C-1-M	30 Mar 2011	Minami-minowa-mura, Nagano	Pa	Pa	S-25, S-89	
S-111		25 Jul 2010	Minami-minowa-mura, Nagano	Pa, Bp, Qs			Ogawa et al. 2018.
	S-244-M	30 Jul 2011	Minami-minowa-mura, Nagano	Pa, Bp, Qs	Qs	S-111	
S-112		25 Jul 2010	Minami-minowa-mura, Nagano	Pa, Pd			
S-1~3		23 Jul 2009	Ooshika-mura, Nagano	Qs，Ts			
S-10~13		20 Jul 2009	Nakagawa-mura, Nagano	Qa, Qs			Ogawa et al. 2018.
S-235		6 Aug 2009	Nakagawa-mura, Nagano	Qa, Qs			
	S-235-M	22 Jul 2011	Nakagawa-mura, Nagano	Qa, Qs	Qs	S-235	
S-236		6 Aug 2009	Nakagawa-mura, Nagano	Qs, Pd			
	S-236-M	22 Jul 2011	Nakagawa-mura, Nagano	Qs, Pd	Qs	S-236	
S-18~20		23 Jul 2009	Matsukawa-machi, Nagano	Pd, Qs			
	S-203-M	18 Dec 2010	Matsukawa-machi, Nagano	Pd, Qs	Qs	S-18	
	C-28-M	10 Aug 2011	Ootaki-mura, Nagano	Qc	Qc	C-28[b]	
S-221		11 Jul 2011	Kanazawa-shi, Ishikawa	Pd			
	C-9-M	26 Jun 2011	Kyoto-shi, Kyoto	Pd	Pd	C-9[b]	
	C-11-M	26 Jun 2011	Kyoto-shi, Kyoto	Pd	Pd	C-11[b]	
C-2		15 May 2011	Kumejima Island, Okinawa	Pl			Ogawa et al. 2018.
	C-2-M	15 May 2011	Kumejima Island, Okinawa	Pl	Pl	C-2	

[a] Bp: *Betula platyphylla* var. *japonica*, Pa: *Picea abies*, Pd: *Pinus densiflora*, Pl: *Pinus luchuensis*, Pt: *Pinus thunbergii*, Qa: *Quercus acutissima*,Qc: *Quercus crispula*, Qs: *Quercus serrata*, Ts: *Tsuga sieboldii.*

[b] Basidioma specimens were sampled on the same date of ectomycorrhizal sampling, except for the case of 8508 that was only sampled at post ectomycorrhizal sampling on 1th Oct 2011. ND: Basidioma sample was not stored as dry specimens.

Table 2 Culture establishment of *Cantharellus anzutake* from basidiomata.

Specimen ID	Medium used for isolation[a]	Number of basidioma tissue		Established culture ID	Sequence data deposited to DDBJ	
		Tested	Recovered		ITS2	*tef-1*
S-10	MNC+Ch100	10	1	EN-51 (=NBRC-113266)	LC368815	LC368819
S-89	MNC+St100, Te50	15	1	EN-52 (=NBRC-113267)	LC368812	LC368820
	MNC+St100, Pe100	15	0			
	MNC+ PCN200	15	0			
S-111	MNC+St100, Te50	8	1	EN-53 (=NBRC-113268)	LC368813	
	MNC+St100, PCN100	2	0			
	MNC+PCN200	2	0			
S-112	MNC+St100, Te50	4	0			
	MNC+St100, Pe100	3	0			
	MNC+ PCN200	3	0			
S-221	MNC+St100, Te50	5	0			
C-2	MNC+St100, Te50	35	0			
S-1~3	MNC+Ch100	15	0			
	WA	15	0			
S-11~13, 18~20, 25, 235, 236	MNC+Ch200	90[b]	0			

[a] Ch: Chloramphenicol, St: Streptomycin, Te: Tetracycline, PCN: Penicillin, WA: water agar. Numerical values (50–200) following abbreviations mean concentrations (mg/L) of those antibiotics in the MNC agar.
[b] Each of the 9 specimens tested 10 basidoma tissue.

Table 3 Culture establishment of *Cantharellus anzutake* from ectomycorrrhizae.

Mycorrhizal sample ID	Host plant[a]	Number of ectomycorrhizal segments		Isolation ratio (%)	Established culture ID	Sequence data deposited to DDBJ	
		Tested	Recovered			ITS-2	*tef-1*
S-201-M	Pa	10	5	50.0	EN-60 (=NBRC-113269) EN-61 (=NBRC-113270)		LC368816 LC368817
C-1-M	Pa	21	10	47.6	C-1[b]		
Average for *Picea*				48.8			
C-2-M	Pl	47	23	48.9	C-2		
C-9-M	Pd	22	0	0			
C-11-M	Pd	22	1	4.5	C-11[b]		
C-12-M	Pd	30	7	23.3	C-12		
C-18-M	Pd	30	14	46.7	C-18		
C-19-M	Pd	30	0	0			
C-30-M	Pt	30	7	23.3	C-30		
S-210-M	Pd	10	5	50.0	EN-68, EN-69		
Average for *Pinus*				12.2			
S-203-M	Qs	10	5	50.0	EN-97		
S-235-M	Qs	31	2	6.5	S-235		
S-236-M	Qs	10	5	50.0	EN-98		
S-244-M	Qs	30	5	16.7	S-244		
C-28-M	Qc	30	1	3.3	C-28[b]		
C-23-M	Qc	30	26	86.7	C-23 (=NBRC-113265)	LC368814	LC368818
C-25-M	Qc	30	0	0			
C-26-M	Qc	30	4	13.3	C-26		
Average for *Quercus*				28.3			

[a] Pa: *Picea abies*, Pd: *Pinus densiflora*, Pl: *Pinus luchuensis*, Pt: *Pinus thunbergii*, Qc: *Quercus crispula*, Qs: *Quercus serrata*

[b] Cultures were successfully established and identified to species level but were not stored.

Supplementary Table 1 Comparison of *Cantharellus anzutake* isolation from basidiomata and ectomycorrhizae.

Paired samples between basidomata and ectomycorrhizae	Basidiomata			Ectomycorrhizae		
	Number of tissue		Isolation ratio	Number of segments		Isolation ratio
	Tested	Recovered		Tested	Recovered	
S-25 and S-89 vs. S-201-M and C-1-M	55	1	0.018	31	15	0.484
S-111 vs. S-244-M	12	1	0.083	30	5	0.167
S-235 vs. S-235-M	10	0	0	31	2	0.065
S-236 vs. S-236-M	10	0	0	10	5	0.500
S-18 vs. S-203-M	10	0	0	10	5	0.500
C-2 vs. C-2-M	35	0	0	47	23	0.489
			0.017 (0.014)[a]			0.368 (0.081)[a]

[a] The value indicates mean with standard error in parenthesis (n=6), which shows significant difference between basidiomata and ectomycorrhizae at P=0.007 by the t-test. The numerical data of isolation ratio were compared between basidiomata and ectomycorrhizae after the arc sine transformation.