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Phylogenetic analyses of Japanese golden chanterelles and a new species description, *Cantharellus anzutake* sp. nov.

Wakana Ogawa [a], Naoki Endo [a, b], Masaki Fukuda [a, c], Akiyoshi Yamada [a, c, d, *]

[a] Department of Bioscience and Food Production Science, Interdisciplinary Graduate School of Science and Technology, Shinshu University, 8304, Minami-minowa, Nagano, 399-4598, Japan
[b] Fungus/Mushroom Resource and Research Center, Faculty of Agriculture, Tottori University, 4-101 Koyama, Tottori, 680-8553, Japan
[c] Department of Bioscience and Biotechnology, Faculty of Agriculture, Shinshu University, 8304, Minami-minowa, Nagano, 399-4598, Japan
[d] Division of Terrestrial Ecosystem, Institute of Mountain Science, Shinshu University, 8304, Minami-minowa, Nagano, 399-4598, Japan

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ABSTRACT

The Japanese golden chanterelle commonly identified as *Cantharellus cibarius* was sampled in a broad range of forest vegetation. A total of 90 fresh and 11 herbarium specimens were examined microscopically, subjected to sequencing analysis of their nuclear ribosomal RNA (rDNA) and *tef-1* genes, and their characteristics were compared with those of European *C. cibarius*. Based on morphological and ecological characteristics, basidioma samples from Japan were divided into four species. While specimens of *Cantharellus* sp. 4 from Hokkaido Island were included in the European *C. cibarius* clade phylogenetically, the other three species formed three unique clades. Among these, *Cantharellus anzutake* sp. nov. is sister to the clade of *C. cibarius* and was widely sampled from the northern limit of Honshu Island to the southern limit of Kumejima Island in Ryukyu Islands. Although *C. anzutake* was morphologically similar to *C. cibarius*, the two species were phylogenetically distinct. Other morphologically similar but genetically distinct chanterelle species from India exhibited macroscopic and microscopic differences compared with *C. anzutake*.

1. Introduction

Chanterelles are one of the most important edible ectomycorrhizal mushrooms in the world, with a large market share. Especially, *Cantharellus cibarius* Fr. and several similar species known as yellow chanterelles have high commercial value, likely in excess of a billion dollars annually (Hall, Wang, & Amicucci, 2003; Watling, 1997). Therefore, it is important to identify true species of chanterelles for market supply. However, their taxonomy remains confusing. Over 400 species of *Cantharellus* (Hydnaceae, Cantharellales; Hibbett et al., 2014) were described by the end of the 20th century, but only approximately 60 species were considered valid (Eyssartier & Buyck, 2000). Over the past 15 y, approximately 70 new *Cantharellus* species have been described (Buyck, 2016), and approximately 130 *Cantharellus* species are considered valid (Buyck, 2016; Buyck, Kauff, Eyssartier, Couloux, & Hofstetter, 2014). *Cantharellus cibarius*, the type species of this genus, was first described by Fries (1821), as follows: "vitellinus, pileo carnoso subrepando glabro, plicis tumidis, stipites solido deorsum attenuato." As the description is vague and could include many chanterelle species, a more detailed description of *C. cibarius* by epitypification was adopted recently (Olariaga et al., 2016). Several fleshy yellow and more or less closely related chanterelle species have been described based on staining upon bruising, size, and shape of basidiomata, as well as on the ecology and habitat of the species (Buyck et al., 2016b; De Kesel et al., 2016), all of which approximately match the description by Fries (1821). Recently, molecular methods, such as sequence comparisons of the nuclear ribosomal RNA (nrDNA) or translation elongation factor EF1-alpha (*tef-1*) genes, have been applied to identify *C. cibarius* sensu stricto and its related species (Buyck, Hofstetter, & Olariaga, 2016a, b, c).

Cantharellus cibarius sensu lato was previously believed to have a cosmopolitan distribution not only in the Northern Hemisphere, but also in Central Africa (Pilz, Norvell, Danell, & Molina, 2003). Therefore, many varieties and forms of *C. cibarius* have been

* Corresponding author. Department of Bioscience and Biotechnology, Faculty of Agriculture, Shinshu University, 8304, Minami-minowa, Nagano, 399-4598, Japan.
E-mail address: akiyosh@shinshu-u.ac.jp (A. Yamada).

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described, all essentially from Europe and North America (Eyssartier & Buyck, 2000; Hansen & Knudsen, 1997; Pegler, Roberts, & Spooner, 1997; Persson, 1997; Pilz et al., 2003). Recent molecular phylogenetic studies of yellow chanterelles revealed their true nature (Buyck et al., 2016b; Olariaga et al., 2016). European golden chanterelle, i.e., *C. cibarius* sensu stricto, is distributed mainly in the northern and central parts of Europe (Buyck et al., 2014; Olariaga et al., 2016). In addition, several species of fleshy yellow chanterelles, e.g., *Cantharellus roseocanus* Redhead, Norvell & Moncalvo, *Cantharellus cascadensis* Dunham, O'Dell & R. Molina, *Cantharellus formosus* Corner, *Cantharellus californicus* Arora & Dunham, and *Cantharellus tenuithrix* Buyck & V. Hofst., have been described from North America over the past two decades, most of which were previously considered as conspecific to European *C. cibarius* (Buyck et al., 2016a, b, c, 2011; Arora & Dunham, 2009; Buyck & Hofstetter, 2011; Dunham, Kretzer, & Pfrender, 2003a, b; Foltz, Perez, & Volk, 2013; Leacock et al., 2016; Redhead, 2012, p. 1). In Asia, three new species, *Cantharellus applanatus* Deepika, Ram. Upadhyay & Mod.S. Reddy, *Cantharellus elongatipes* Deepika, Ram. Upadhyay & Mod.S. Reddy, and *Cantharellus natarajanii* Ram. Upadhyay & Mod.S. Reddy, were described from India as cryptic species morphologically similar but distinguishable from European *C. cibarius* (Deepika, Reddy, & Upadhyay, 2014). In addition, two endemic, fleshy, yellow, and ridged hymenium *Cantharellus* species, possibly belonging to the subgenus *Cantharellus*, have been described from China (Shao, Buyck, Tian, Liu, & Geng, 2016a, b, 2011; Tian, Buyck, Shao, Pei-Gui Liu, & Fang, 2012): *Cantharellus yunnanensis* W.F. Chiu (Chiu, 1973) and *Cantharellus tuberculosporus* M. Zang (Zang, 1980). However, *C. cibarius* s.s. and its closely related species cannot be distinguished from one another based on morphological features alone (Arora & Dunham, 2009; Buyck et al., 2016b; Dunham et al., 2003a, b; Feibelman, Bennett, & Cibula, 1996, 1994; Olariaga et al., 2015, 2016).

In Japan, *C. cibarius* was first reported by Hennings (1900) without providing any morphological description or specimen designation. The species description was first conducted by Kawamura (1908) based on a specimen sampled in Jul 1908 under a *Pinus densiflora* Sieb. & Zucc. forest of Mt. Eimeiji-yama, Nagano Prefecture, in the central region of Honshu Island. Unfortunately, the described specimen was lost after the death of Kawamura in 1946. Since then, the Japanese fleshy and yellowish chanterelles sampled in Hokkaido, Honshu, Shikoku, Kyushu Islands, and Ryukyu Islands have been identified consistently as *C. cibarius* (Imazeki & Hongo, 1989; Ito, 1955; Katsumoto, 2010; Kawamura, 1955; Tanabe & Ogawa, 2015).

It is generally known that the islands of Japan range in latitude between N 25–45° and host varied forest vegetation, from boreal and subalpine in Hokkaido Island to subtropical and tropical in Ryukyu Islands (Peel, Finlayson, & McMahon, 2007). Therefore, we hypothesized that the known Japanese *C. cibarius* population includes some cryptic species, as has been noted among North American yellow–golden chanterelles. In fact, the fruiting season of Japanese *C. cibarius* differs according to the geographic region: April and May in Ryukyu Islands (range of maximum temperature: 25–30 °C), Jul to Oct in Nagano (20–30 °C), and Sep to Oct in Hokkaido (15–25 °C). Therefore, it is important to classify Japanese fleshy yellowish chanterelles based on their ecology, morphology, and molecular phylogeny. In the present study, we examined the taxonomy of a probable species complex of known Japanese *C. cibarius* based on macroscopic and microscopic morphological observations and molecular phylogenetic analysis. Here, we present the nature of the Japanese *C. cibarius* species complex and describe a new Japanese golden chanterelle species.

2. Materials and methods

2.1. Specimens examined

Basidiomata of fleshy yellowish chanterelles were collected from various forest sites in Japan from 2010 to 2014 (Table 1). Fresh Swedish *C. cibarius* basidiomata specimens were gifts from Niclas Bergius. The macroscopic features of these basidiomata, such as the size, shape, color, and texture, were recorded. They were then freeze-dried, oven-dried at 70 °C for one night to inactivate DNase and other oxidative enzymes, and stored in the laboratory as dried specimens. In addition, dried voucher specimens of Japanese *C. cibarius* were obtained from the herbaria of the Rishiri Town Museum (RTMFU), Natural History Museum of Hokkaido University (SAPA), National Museum of Natural Science, Tokyo (TNS), and Tottori Mycological Institute (TMI).

2.2. Microscopic observations of selected specimens

A portion of hymenium tissue was rehydrated in a drop of 1% KOH and then in distilled water for 1 h to prepare basidiospores for microscopy, which were mounted with lactic acid on a slide and observed under a differential interference contrast microscope (AXIO Imager A1, Carl Zeiss Inc., Göttingen) using an immersion ×100 objective lens. Preparation of specimens for microscopic examination and description of hyphal structure followed Largent, Johnson, and Watling (1977) and Clémençon (2009, 2012). Fluorescent microscopic analysis was performed to assess the presence/absence of autofluorescence of hyphal structures using UV irradiation (Agerer, 1990). Some samples from dried specimens were preliminarily rehydrated in 70% ethanol and subsequently in distilled water to confirm the effects of the KOH solution on the wall structure and some fragile elements on and inside the cells. Fifty spores of each specimen were measured for their length, width, and shape (length/width). Basidia, pileipellis, hymenophoral trama, and stipitipellis were examined microscopically and measured. Numerical data of the microscopic structures were measured using Image-J software after taking a photograph of the structures. The obtained numerical data were statistically analyzed using one-way ANOVA or Student's *t*-test with Kaleida Graph ver. 4.0 (Hulinks, Tokyo). Morphological identification of Japanese *C. cibarius* sensu Kawamura was performed as described by Kawamura (1955) and Imazeki and Hongo (1989). Two voucher specimens, C-84 (TNS-F-61926) and C-85 (TNS-F-61927), were deposited in the herbarium of the National Museum of Nature and Science, Tokyo, Japan.

2.3. Molecular phylogenetic analyses

Genomic DNA was extracted from basidioma specimens as described by Gardes and Bruns (1993) with minor modifications. Briefly, a portion of the extracted total DNA was used for PCR amplification of the internal transcribed spacer (ITS) region, the large subunit (LSU) locus of nrDNA, and the *tef-1* locus. The ITS region including 5.8S rDNA was amplified using the previously described primer ITS1F (Gardes & Bruns, 1993) and the novel *Cantharellus*-specific primer C28S (5′-cactgacggcctattgtactt-3′). The LSU locus was amplified using the novel *Cantharellus*-specific primers C2F (5′-tgaccgtcataggtgctttg-3′) and Tw14 (White, Bruns, Lee, & Taylor, 1990). The *tef-1* locus was amplified as described by Morehouse et al. (2003). PCR amplifications were conducted using the GeneAmp PCR System 2700 (Applied Biosystems, Foster). The 25 μL PCR mixture consisted of 2.5 μL 10× DreamTaq buffer, 2.5 μL 0.2 mM dNTP, 2.5 μL 0.5 μM each primer, 0.125 μL 0.625 U

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