Journal of Plant Research (2021) 134:1213–1224
https://doi.org/10.1007/s10265-021-01337-w

REGULAR PAPER – ECOLOGY/ECOPHYSIOLOGY/ENVIRONMENTAL BIOLOGY

Mycobiont diversity and first evidence of mixotrophy associated with Psathyrellaceae fungi in the chlorophyllous orchid *Cremastra variabilis*

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Received: 11 April 2021 / Accepted: 10 August 2021 / Published online: 17 August 2021

Abstract

Mixotrophy (MX, also called partial mycoheterotrophy) in plants is characterized by isotopic abundances that differ from those of autotrophs. Previous studies have evaluated mycoheterotrophy in MX plants associated with fungi of similar ecological characteristics, but little is known about the differences in the relative abundances of ^{13}C and ^{15}N in an orchid species that associates with several different mycobionts species. Since the chlorophyllous orchid *Cremastra variabilis* Nakai associates with various fungi with different ecologies, we hypothesized that it may change its relative abundances of ^{13}C and ^{15}N depending on the associated mycobionts. We investigated mycobiont diversity in the chlorophyllous orchid *C. variabilis* together with the relative abundance of ^{13}C and ^{15}N and morphological underground differentiation (presence or absence of a mycorhizome with fungal colonization). Rhizoctonias (Tulasnellaceae, Ceratobasidiaceae, Sebacinales) were detected as the main mycobionts. High differences in $\delta^{13}C$ values (– 34.7 to – 27.4 ‰) among individuals were found, in which the individuals associated with specific Psathyrellaceae showed significantly high relative abundance of ^{13}C. In addition, Psathyrellaceae fungi were always detected on individuals with mycorhizomes. In the present study, MX orchid association with non-rhizoctonia saprobic fungi was confirmed, and the influence of mycobionts on morphological development and on relative abundance of ^{13}C and ^{15}N was discovered. *Cremastra variabilis* may increase opportunities to gain nutrients from diverse partners, in a bet-hedging plasticity that allows colonization of various environmental conditions.

Keywords Mycorrhizae · Mycorhizomes · Orchid morphology · Partial mycoheterotrophy · Psathyrellaceae · Rhizoctonias

Introduction

Mixotrophy with mycorrhizal fungi (MX, also called partial mycoheterotrophy) is a nutritional mode with two carbon sources (Gebauer and Meyer 2003; Selosse and Roy 2009) in which plants gain organic matter from their photosynthesis (autotrophy) and from their mycobionts (a nutrition called mycoheterotrophy). This physiological feature was identified due to the survival of achlorophyllous mutants (Julou et al. 2005; Selosse et al. 2004) and to unusual stable isotope natural abundance in MX plants (Gebauer and Meyer 2003; Hynson et al. 2013; Julou et al. 2005; Selosse and Roy 2009). Considering the latter point, the natural isotopic abundance of the biomass gained mycoheterotrophically is often enriched in ^{13}C and ^{15}N compared with that of purely autotrophic C_3 plants, at least in most MX plants associated with Asco– and Basidiomycetes (Hynson et al. 2013).

Analysis of mycoheterotrophic plants demonstrates that the relative abundance of ^{13}C is somewhat higher, while that

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of ^{15}N is lower when the associated fungi have a saprobic life in soils than when they form ectomycorrhizal symbioses with surrounding trees (Hynson et al. 2013; Lee et al. 2015; Martos et al. 2009; Ogura-Tsujita et al. 2009). In recent study, metabolism of older carbon sources fixed ca. 10–40 years by mycoheterotrophic orchid exploiting saprobic fungi were revealed by $\Delta^{14}C$ analyses (Hatté et al. 2020; Suetsugu et al. 2020a). In MX species associating with ectomycorrhizal fungi, the relative abundances of ^{13}C and ^{15}N are typically intermediate between those of autotrophic plants and mycoheterotrophic plants or fungal fruit bodies (Bidartondo et al. 2004; Julou et al. 2005; Motomura et al. 2010; Selosse and Roy 2009; Yagame et al. 2011). Additionally, carbon heterotrophy level can be estimated by a linear mixing-model calculation using the relative abundance of ^{13}C in MX compared to that of mycoheterotrophic and autotrophic plants from the same site (Gebauer and Meyer 2003; Hynson et al. 2013). The possibility of MX nutrition in chlorophyllous orchids associated with rhizoctonia, and symbiotic fungi of the usual orchid that are saprobic and endophytic in non-orchid roots (Tulasnellaceae, Ceratobasidiaceae and Serendipitaceae; Dearnaley et al. 2013a; Selosse and Martos 2014), is questionable. Indeed, their relative abundances of ^{15}N and ^{2}H suggest possible MX abilities, called 'cryptic mycoheterotrophy' (Selosse and Martos 2014; Gebauer et al. 2016; Schiebold et al. 2018; Schweiger et al. 2018).

Stable isotope analysis and molecular identification of mycobionts have become standard tools for research on trophic strategies in plants (Hynson et al. 2013; Schiebold et al. 2017; Selosse et al. 2017). Mycobionts of candidate MX plants in the Burmanniaceae and Gentinaceae have not been identified (Bolin et al. 2015; Cameron and Bolin 2010), but mycobionts in these families usually belong to the Glomeraceae (Merckx et al. 2012). Mixotrophic feature was investigated by estimation of values of $\delta^{13}C$ and $\delta^{15}N$ between grassland herbaceous plant, *Pterygocalyx volubilis* Maxim. in Gentianaceae, neighboring C_3 plants and spore of arbuscular mycorrhizal fungi, in which transfer of carbon from surrounding C_3 plants via hyphal networks were suggested (Suetsugu et al. 2020b). Such physiological features with arbuscular mycorrhizal fungi were also found in Pteridophyta (*Ophioglossum* spp.) in a network with Poaceae (Suetsugu et al. 2020c). Mixotrophic features in the Ericaceae and Orchidaceae were also confirmed, with Asco- and Basidiomycetes (Hynson et al. 2009; Lallemand et al. 2017, 2018; Zimmer et al. 2007). The MX subfamily Pyroleae spp. in the Ericaceae is mainly associated with ectomycorrhizal fungi (Hashimoto et al. 2012; Matsuda et al. 2012; Tedersoo et al. 2007). In orchids, autotrophic and possibly cryptic mixotrophic species associate with rhizoctonias (Selosse and Martos 2014). Achlorophyllous and MX species associate with ectomycorrhizal basidiomycetes and ascomycetes such as the Thelephoraceae, Russulaceae, Sebacinaceae or Tuberaceae (e.g. Bidartondo et al. 2004; Ogura-Tsujita et al. 2012; Selosse et al. 2004). In addition, some mycoheterotrophic orchids associate with saprobic fungi such as *Psathyrella* spp. (Psathyrellaceae) or *Mycena* spp. (Mycenaceae; Martos et al. 2009; Ogura-Tsujita et al. 2009; Selosse et al. 2010), but no MX orchid associating with non-rhizoctonia saprobic fungi, such as Psathyrellaceae, Mycenaceae or Tricholomataceae, has been reported so far.

Orchids are one of the largest angiosperm families, with > 28,000 species (Christenhusz and Byng 2016). They produce minute seeds with an undifferentiated embryo and no endosperm. Germination of these seeds depends on fungi and their juvenile development with mycobionts is initially mycoheterotrophic (Leake 1994; Merckx et al. 2013). At adulthood, after initial mycoheterotrophy, plants either keep their mycoheterotrophy or develop photosynthetic organs allowing either autotrophic or MX nutrition. Mixotrophy is characterized by dynamic isotopic features. Lower abundances of ^{13}C were found in Ericaceae and Orchidaceae species under higher light conditions (e.g. Gonneau et al. 2014; Matsuda et al. 2012; Preiss et al. 2010), suggesting higher mycoheterotrophy in shaded conditions. Available mycobionts also drive the relative abundance of ^{15}N in MX *Epipactis* species, e.g. with higher relative abundance of ^{15}N in association with ectomycorrhizal ascomycetes than with ectomycorrhizal basidiomycetes (Schiebold et al. 2017). However, little is known about how each mycobiont influences the relative abundances of ^{13}C and ^{15}N of orchids associating with several fungi with diverging ecology.

The orchid genus *Cremastra* (tribe Calypsoeae) comprises seven species (Yukawa 1999). Among them, *Cremastra variabilis* Nakai (= *Cremastra appendiculata* (D.Don) Makino in a previous study; Yagame et al. 2013) is a common and widely distributed chlorophyllous species, found from Japan and Sakhalin Island in Russia, to Taiwan and the Himalayas (Maekawa 1971). It occurs in dry ridge lines and wet sites along small streams in various forests of ectomycorrhizal and arbuscular mycorrhizal trees. Previous studies have shown that it has diverse fungal partners. Saprobic Psathyrellaceae isolated from *C. variabilis* mycorhizomes (rhizome colonized by fungi) induced seed germination and mycorhizome development in vitro (Yagame et al. 2013). However, rhizoctonias (namely Tulasnellaceae, Ceratobasidiaceae, Serendipitaceae) were also detected from the roots of adult *C. variabilis* collected from habitats in other preliminary investigations (T. Yagame unpublished data). From these results, an intriguing possibility is that *C. variabilis* has a nutrition intermediate between autotrophy and mycoheterotrophy, and a variable isotopic values depending on its dominant mycobionts.

We have investigated morphological and physiological features of MX candidate orchid associating with

non-rhizoctonia saprobic fungi, focusing on (1) mycobiont diversity and morphological features (presence of a mycorhizome), and (2) relative abundances of stable isotopes, depending on mycobionts colonizing the plant and surrounding forest type.

Materials and methods

Sample collection

In total, 57 individuals of *C. variabilis* were collected from 18 sampling sites in Japan (Fig. 1; see GPS data and dominant trees and sampling date in Table 1) for molecular identification of mycobionts, among which 22 individuals with green leaves (20–25 cm length, 3–5 cm wide) from six sites were used for measurement of the relative abundances of ^{13}C and ^{15}N. In a preliminary work, mycorrhizal symbiosis between various fungi and *C. variabilis* were revealed (T. Yagame unpublished data), we assumed that this orchid may change its stable isotope content with fungal partners, due to dominant trees of their habitats. In order to confirm relationship between stable isotope content, fungal partners and dominant trees in the habitat, the samples from the six sites were collected from three ectomycorrhizal and arbuscular forest (Saji, Aso, Nagano) and three purely arbuscular forest (Amegtakiji, Tokumaru, Kamuikotan; Fig. 1; Table 1). In the Tottori Prefecture, where this orchid was found under various vegetation types under similar climatic condition, a special sampling effort was made with collection of 38 individuals from 12 sites to reveal relationships between vegetation types and mycobiont diversity (Fig. 1; Table 1). All individuals were collected at a similar developmental stage, i.e. flowering size with formation of 2–3 corms (i.e. the swollen underground shoots storing water, amino acids and polysaccharides; Fig. 2). This orchid starts to develop a leaf and corm from August to October every year, and the leaf defoliates in May to June of the next year. Because new corm forms underground every year, the number of corms precisely indicates the age of each individual. For isotopes, in addition to leaf samples of this species, autotrophic or mycoheterotrophic plant species growing at the same light level and same distance from soil were also collected as references at each site (5 leaves or shoots per species for 21 species in all; see Table S1 and below). As mycoheterotrophic plants were not available except for the site of Saji (see Table S1), we further collected fruit bodies of eight saprobic fungal species as a reference for saprobic fungal biomass (five samples per species; Table S1). All samples were placed in individual plastic bags, kept at 4 °C, and processed within 1 day after sampling for identification of mycobionts. For measurement of stable isotope values, samples were dried at 60 °C for 4 days and then kept with silica gel.

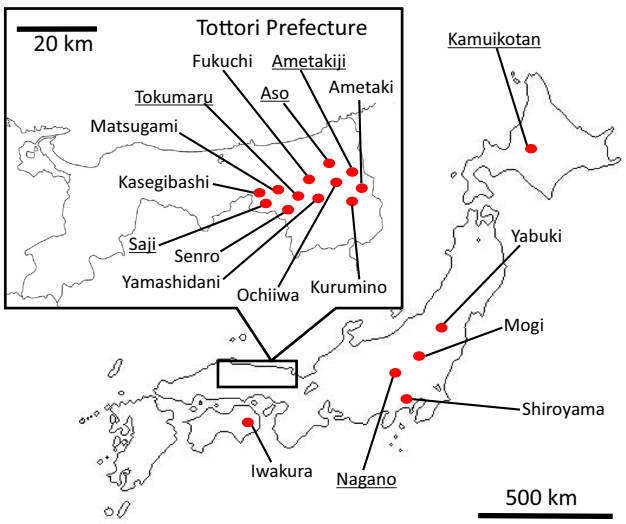

Fig. 1 Dots indicate the locations in Japan of the 18 sites of *Cremastra variabilis* sampled in this study. Sites names of sample collection both of mycobionts diversity and stable isotope values estimation are underlined

DNA extraction and amplification

The roots and rhizomes displaying mycobiont colonization (mycorhizomes) were washed in tap water and hand-sectioned for examination by microscopy in order to confirm fungal colonization. Three confirmed mycorrhizal root sites (ca. 3 cm length) from three roots and one mycorhizome (when available, ca. 1 cm length) were collected from each individual for extraction of DNA. Total DNA was extracted from samples by the cetyltrimethylammonium bromide method (Weising et al. 1995). After additional purification using the Mag Extractor Plant Genome Kit (Toyobo, Osaka, Japan), DNA was dissolved in 50 µL of Tris–EDTA buffer for PCR. The primer pair ITS1F/ITS4 was used to amplify the internal transcribed spacer (ITS) regions of the fungal nuclear ribosomal RNA gene (Gardes and Bruns 1993). The PCR mixture contained 1 µL of the extracted DNA solution, 0.75 U of TaKaRa Ex Taq polymerase (TaKaRa, Otsu, Japan), 0.25 µM of each primer, 200 µM of each deoxynucleotide triphosphate, and 3 µL of the PCR Ex Taq buffer supplied by the Taq provider, in a total volume of 30 µL. PCR was performed using the Program Temp Control System PC-818S (Astec, Fukuoka, Japan) with an initial denaturation step at 94 °C for 2 min, followed by 35 cycles at 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 1 min, and a final elongation step at 72 °C for 10 min. Since the ITS1F/ITS4 primer pair fails to amplify most Tulasnellaceae (Basidiomycota), which are common mycobionts of orchids (Dearnaley et al. 2013a; Yukawa et al. 2009), an additional PCR was performed with the primer

Table 1 *Cremastra variabilis* sampling information

Sampling site Prefecture in Japan	Site name	Site code	Number of collected individuals		Latitude/longitude	Altitude (m)	Sampling date	Mycorrhizal types of forest trees	Dominant trees in the habitat
			With rhizome	No rhizome formation					
	Matsugami	MA	0	2	35 26′ 03″ N, 134 07 47″ E	175	10 Jun. 2011	Arbuscular	*Cryptomeria japonica, Aucuba japonica*
	Saji	SA	0	4	35 20′ 03″ N, 134 08 36″ E	158	10 Jun. 2011	Ectomycorrhizal/ Arbuscular	*Castanopsis sieboldii, Cryptomeria japonica, Euptelea polyandra*
	Kasegibashi	KA	0	5	35 19′ 56″ N, 134 07 48″ E	202	10 Jun. 2011	Arbuscular	*Cryptomeria japonica*
	Senro	SE	3	2	35 21′ 39″ N, 134 12 48″ E	82	10 Jun. 2011	Ectomycorrhizal/ Arbuscular	*Carpinus tschonoskii, Chamaecyparis obtusa*
Tottori Pref	Ametakiji	AJ	0	2	35 29′ 49″ N, 134 23 13″ E	351	10 Jun. 2011	Arbuscular	*Acer sieboldianum, Swida macrophylla*
	Ametaki	AM	0	3	35 28′ 40″ N, 134 24 11″ E	491	10 Jun. 2011	Arbuscular	*Euptelea polyandra*
	Fukuchi	FU	1	2	35 24′ 44″ N, 134 20 22″ E	255	10 Jun. 2011	Arbuscular	*Aesculus turbinata, Cryptomeria japonica*
	Aso	AS	0	5	35 23′ 58″ N, 134 20 54″ E	211	10 Jun. 2011	Ectomycorrhizal/ Arbuscular	*Carpinus japonica, Mallotus japonicus, Quercus glauca*
	Ochiiwa	OT	0	2	35 24′ 24″ N, 134 21 44″ E	274	10 Jun. 2011	Arbuscular	*Acer sieboldianum*
	Yamashidani	YA	0	3	35 23′ 40″ N, 134 21 10″ E	277	10 Jun. 2011	Arbuscular	*Cryptomeria japonica, Zelkova serrata*
	Tokumaru	TO	0	2	35 22′ 11″ N, 134 20 08″ E	166	16 Jun. 2011	Arbuscular	*Cryptomeria japonica, Zelkova serrata*
	Kurumino	KU	0	2	35 22′ 40″ N, 134 24 12″ E	301	16 Jun. 2011	Arbuscular	*Acer pictum, Aesculus turbinata, Cryptomeria japonica*
Nagano Pref	Nagano	NA	2	4	36 05′ 21″ N, 137 57 25″ E	784	24 May 2012	Ectomycorrhizal/ Arbuscular	*Larix kaempferi Pterocarya rhoifolia, Robinia pseudoacacia*
Fukushima Pref	Yabuki	FY	0	4	37 11′ 55″ N, 140 20 57″ E	108	20 Sep. 2011	Arbuscular	*Cryptomeria japonica, Zelkova serrata*

Table 1 (continued)

Sampling site Prefecture in Japan	Site name	Site code	Number of collected individuals		Latitude/longitude	Altitude (m)	Sampling date	Mycorrhizal types of forest trees	Dominant trees in the habitat
			With rhizome	No rhizome formation					
Tokushima Pref	Iwakura	IW	0	1	33 50′ 39″ N, 134 10′ 17″ E	851	17 Oct. 2013	Arbuscular	*Cryptomeria japonica, Zelkova serrata*
Hokkaido Pref	Kamuikotan	KK	0	3	43 44′ 02″ N, 142 12′ 02″ E	107	17 Oct. 2014	Arbuscular	*Cryptomeria japonica, Zelkova serrata*
Kanagawa Pref	Shiroyama	KS	0	2	35 35′ 17″ N, 139 17′ 28″ E	219	17 Sep. 2011	Arbuscular	*Cryptomeria japonica, Zelkova serrata*
Tochigi Pref	Mogi	OM	0	3	36 31′ 49″ N, 140 11′ 20″ E	300	13 Jun. 2014	Arbuscular	*Cryptomeria japonica, Zelkova serrata*

In total, 57 individuals were collected, in which 6 individuals formed rhizome

pair ITS1-OF/ITS4-OF (Taylor and McCormick 2008) for all samples, using the same PCR conditions and program. All PCR products were cloned using the pGEM-T Easy Vec-

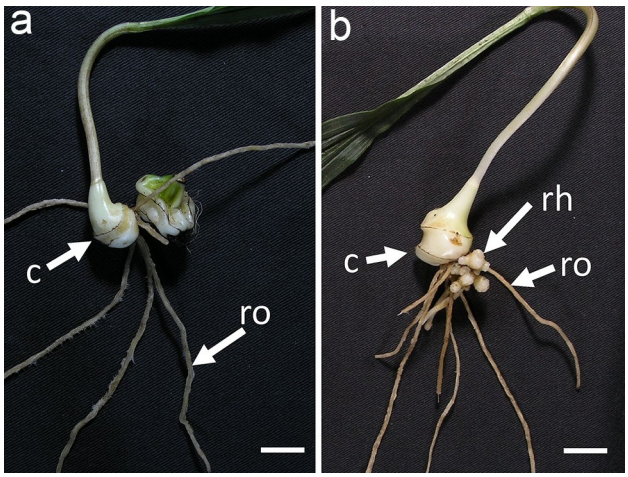

Fig. 2 Root system of *Cremastra variabilis*. **a** Individual without mycorhizome; **b** individual with mycorhizome; **c** corm (base of shoot); *ro* root, *rh* mycorhizome. Bars = 5 cm

torSystem I (Promega, Tokyo, Japan), and three colonies with DNA inserts were arbitrarily selected from each cloning for sequencing by the dideoxy sequencing method, using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) on a Genetic Analyzer 3130 (Applied Biosystems). ITS regions more than 97% identical were regarded as a single operational taxonomic unit (OTU; Nilsson et al. 2008; Stackebrandt and Goebel 1994). All sequence data were deposited in the DNA Data Bank of Japan (DDBJ; Tables S3, Figs. S1–S4; see Supplemental Data with online version of article).

Molecular data and phylogenetic analyses

The sequences obtained were subjected to BLAST searches (Altschul et al. 1997) to determine their taxonomic positions. For influences of forest conditions on mycobionts, the χ^2 test was used to estimate statistical differences between the number of mycobiont species in *C. variabilis* in ectomycorrhizal forests and arbuscular mycorrhizal forests. ITS sequences of Tulasnellaceae, Ceratobasidiaceae, Serendipitaceae, Sebacinaceae and Psathyrellaceae detected in this study were aligned with related sequences downloaded from GenBank, using the program Clustal W version 1.83 (Thompson et al. 1994). Phylogenetic analyses of ITS data sets were conducted individually by the maximum likelihood (ML) method (Strimmer and Haeseler 1996). The best-fit trees were inferred using the GTR + GAMMA analysis model, which was estimated with the program MrModeltest 2.2 (Nylander 2004) in the program PAUP* 4.0b10 (Swofford 2003). To check for statistical support, the bootstrap method (Felsenstein 1985) was applied to the resultant tree topology with 1,000 replicates in the GTR + GAMMA model.

Isotope analysis

The leaf samples for stable isotope measurement were collected 5 × 3 cm (length × width) from the tip of each leaf of *C. variabilis* individuals. All dried samples were ground with 1.1 mm tungsten carbide balls (Biospec Products, Inc., Bartlesville, OK, USA) in 1.5 mL tubes using a Retch MM301 grinder (Retsch Gmbh and Co., Haan, Germany). Stable isotopes of carbon and nitrogen in each sample were measured as described by Tedersoo et al. (2007). The relative abundances of stable isotopes are presented as $\delta^{13}C$ and $\delta^{15}N$ using the equation: $\delta^{15}N$ or $\delta^{13}C = (R_{sample}/R_{standard} - 1) \times 1000$ (‰) , where R is the molar ratio of the stable isotopes, either $^{15}N/^{14}N$ or $^{13}C/^{12}C$. We used an alanine working standard calibrated against a caffeine standard. The $\delta^{13}C$ values are reported relative to the Pee Dee belemnite, and the $\delta^{15}N$ values relative to air. The $\delta^{13}C$ values were tested for normality using a χ^2 test for goodness of fit and the Bartlett test for homogeneity of variances. After testing the isotope abundance datasets for normal distributions and homogeneity of variances, one-way ANOVA was applied to evaluate the statistical differences in δ^{15} N and $\delta^{13}C$ among *C. variabilis,* autotrophic plants and saprobic fruit bodies at three out of six sampling sites, Kamuikotan, Aso and Nagano, and the mean values were then compared by means of Scheffe's f-test. For statistical analyses, Statcel 4 software, OMS Publishing Inc., Saitama, Japan was used and significance level was established at an alpha risk of 5%.

Results

Morphological characteristics and mycorrhizal diversity

Three types of subterranean organs, namely corms, roots and mycorhizomes, were found in *C. variabilis* (Fig. 2). Roots and corms were always present, but we noted an inconsistent presence of mycorhizomes: six individuals from the Aso, Nagano and Ametakiji sites displayed mycorhizomes. The individuals NA-4, NA-5 (from the Nagano site) developed branched mycorhizomes (Fig. 2), while other mycorhizomes (individual NA-6 from the Nagano site, individuals AJ-1, AJ-2 and AJ-3 from the Ametakiji site and individual AS-3 from the Aso site) were not branched and measured less than 2 cm. In all, 50 individuals developed no mycorhizome but only roots. The fungal colonization was limited in the roots and small mycorhizomes, but more abundant in the branched mycorhizomes. *Cremastra variabilis* mycorhizomes only appeared at the Nagano, Ametakiji, and Aso sites where decaying fallen logs displaying white rot occurred in the immediate vicinity of the plants, and its mycorhizomes adhered to decayed wood. At the Nagano site, a big fallen log (length and thickness: ca. 5 m × 40 cm) was found, to which three individuals (NA-4, NA-5, NA-6) adhered. At the Ametakiji and Aso sites, 10–20 thin fallen logs (ca. 30–50 cm × 5–10 cm) were found nearby. All individuals in Aso adhered to the fallen logs, whereas AJ-1 and AJ-2 only adhered to the fallen logs in Ametakiji.

We detected from 1 to 12 fungal sequences from all collected *C. variabilis* individuals (out of 9 or 12 clones sequenced per individual; see Table S2). In total, 422 fungal sequences representing 68 OTUs were gained from 172 mycorrhizal samples (roots and mycorhizomes; 534 clones sequenced; Tables S2, S3). The success rate of obtaining sequences after cloning was thus 79.0%. Rate of detection of identical fungal sequences from at least 3 colonies was 15.3% (29 times out of 189 clone selections). BLAST analyses showed that dominant mycobiont groups (83.1%: 351 out of 422 OTUs): were Ceratobasidiaceae, Sebacinaceae, Serendipitaceae and Tulasnellaceae as well as Psathyrellaceae (Table S2), although other fungi were also occasionally detected (Table S3). In all, these five families respectively accounted for 9.2% (39/422), 7.3% (31/422), 4.5% (19/422), 48.3% (203/422), and 13.9% (59/422) of the sequences obtained. The remaining 16.8% (71/422) were attributed to one Mucoromycete (*Mucoromycotina* sp. GenBank: LC189046; Mucoromycotina UNITE: MK429879), five ascomycetous families including Herpotrichiellaceae, Glomerellaceae and Helotiaceae, as well as seven basidiomycetous families including Marasmiaceae, Physalacriaceae and Hygrophoraceae (Table S3). PCR amplification using ITS1-OF and ITS4-OF primers detected several Tulasnellaceae sequences (phylotypes E, F, G, and H: Table S2) undetected when using ITS1F and ITS4.

The 293 ITS sequences in rhizoctonias (Ceratobasidiaceae, Tulasnellaceae Serendipitaceae, and Sebacinaceae; Table S2) were used to reconstruct molecular phylogenetic trees using the ML method (Figs. S1–S4). Phylotypes A, B, D and E in Ceratobasidiaceae clustered with mycobionts of chlorophyllous orchids, while Ceratobasidiaceae phylotypes F related to plant pathogens (Fig. S1). Detected sequences of Sebacinaceae formed clades with orchid mycorrhizal species that are also endophytic and ectomycorrhizal (Weiss et al. 2016), whereas Serendipitaceae were closely related to ericoid mycobionts (Fig. S2). The nine Tulasnellaceae phylotypes were divided into two large clades in ML analysis, among which phylotypes D, F, G, H and I clustered with orchid mycobionts (Fig. S3). The five detected Psathyrellaceae phylotypes (Fig. S4) displayed similarities to different species: phylotype A matched *Psathyrella candolleana* (Fr.) Maire (100% identity; AB470877) and mycobionts of the mycoheterotrophic orchids *Epipogium roseum* Lindl. and *Eulophia zollingeri* J.J.Sm.; phylotype C was highly similar to *Coprinellus callinus* (M. Lange & A.H. Sm.) Vilgalys, Hopple & Jacq. Johnson (99.4% identity; JF907841);

phylotype E was highly similar to *Coprinellus domesticus* (Bolton) Vilgalys, Hopple & Jacq. Johnson (99.8% identity; AB470877); phylotype D formed a clade with *Coprinellus radians* (Desm.) Vilgalys, Hopple & Jacq. Johnson (96.9% identity; FJ582637); finally, phylotype E clustered with a mycobiont of the mycoheterotrophic *E. roseum* (Yamato et al. 2005).

Regarding correlations between organs and mycobionts, Psathyrellaceae mycobionts were detected on mycorhizomes in all seven individuals with mycorhizomes. The individuals NA-4 and NA-5 with branched mycorhizomes associated with Psathyrellaceae phylotype E only, while NA-6 with non-branched mycorhizome associated with Psathyrellaceae phylotypes A and E, as well as with other non-rhizoctonia fungi (Table S2). Other individuals with small non-branched mycorhizomes (AJ-1, AJ-2, AJ-3 and AS-3) associated with Psathyrellaceae phylotypes B and D, as well as with other fungi including rhizoctonias (Table S2).

In 12 sampled sites centered around Tottori, 3 sites were dominated by ectomycorrhizal trees (Aso, Saji, Senro; Table 1), and in these sites 8 ectomycorrhizal fungal sequences (7 Sebacinaceae, 3 Serendipitaceae Phylotype A and B, one Atheliaceae one *Lactarius*) were detected in 8 out of 11 orchid individuals (Tables S2, S3). In the 9 other Tottori sites, which were dominated by arbuscular mycorrhizal trees (plantation of *Cryptomeria japonica* D.Don.), 35 ectomycorrhizal fungal sequences (17 Sebacinaceae, 16 Serendipitaceae A and B, 2 *Tomentella*, one *Lactarius*) were found in 14 out of 26 individuals (Tables 1, S2, S3). Saprobic rhizoctonia sequences (Ceratobasidiaceae, Serendipitaceae phylotype C and Tulasnellaceae) were detected both from *C. variabilis* collected from ectomycorrhizal forests (12 Ceratobasidiaceae, 3 Serendipitaceae phylotype C and 10 Tulasnellaceae found in 9 out of 12 individuals; Table S2) and arbuscular mycorrhizal forests (22 Ceratobasidiaceae, 12 Serendipitaceae phylotyepe C and 48 Tulasnellaceae found in 19 out of 26 individuals; Table S2). The χ^2 test revealed no statistical differences in mycobiont diversity in *C. variabilis* between ectomycorrhizal forests and arbuscular mycorrhizal forests ($P > 0.05$).

Relative abundances of ^{13}C and ^{15}N

We examined the relative abundances of ^{13}C and ^{15}N in leaves of *C. variabilis* from six sites (Fig. 3). The mean values of $\delta^{15}N$ and $\delta^{13}C$ in *C. variabilis* individuals collected in three sites Tokumaru, Saji and Ametakiji were not compared by the statistical test because of low sampling sizes. The mean values between autotrophic plants and saprobic fungi including two Ericaceae mycoheterotorphic plants (only at Saji) were compared by the statistical test as references for *C.variabilis* (Tables S4, S5).

Regarding $\delta^{13}C$, the highest variations among individuals at single sites were detected at Nagano, from – 31.7 to – 26.2‰, and the lowest variations at Kamuikotan (from –34.4 to –34.3‰; Fig. 3). At Aso, $\delta^{13}C$ values of *C. variabilis* individuals associating Psathyrellaceae phylotype B mycobionts were significantly higher than those of reference autotrophic plants (Table S4; $P < 0.05$), whereas these values of *C. variabilis* at Kamuikotan and Nagano (NA-1, NA-2, NA-3) associating rhizoctonias and Psathyrellaceae phylotype A did not differ significantly from those of autotrophic plants (Table S4; $P > 0.05$). Although no statistical test was applicable, *C. variabilis* individuals associating with Psathyrellaceae phylotype A and rhizoctonias mycobionts at Saji had $\delta^{13}C$ values close to those of reference autotrophic plants and MX species in Ericaceae species, *P. japonica* (Table S4). At Nagano, no significant differences in $\delta^{13}C$ values were found between three *C. variabilis* individuals (NA-1, NA-2, NA-3) associating with Psathyrellaceae phylotype A and rhizoctonias and autotrophic plants. Yet, the mean value of three individuals (NA-4, NA-5, NA-6) having Psathyrellaceae phylotype E mycobiont was significantly higher than that of autotrophic plants and that of individuals (NA-1, NA-2, NA-3) associating with Psathyrellaceae phylotype A and rhizoctonias (Table S4; $P < 0.05$). Thus, a correlation between the relative abundance of ^{13}C and association of Psathyrellaceae phylotype E and B mycobiont was evidenced at Nagano and Aso, although 2 individuals (AJ-1, AJ-2) associating Psathyrellaceae phylotype B did not allow statistical tests in Ametakiji.

The highest variations in $\delta^{15}N$ among individuals were detected at Ametakiji (from – 0.7 to 1.8‰), while the lowest variations were found at Tokumaru (from – 1.0 to – 0.7‰; Fig. 3). $\delta^{15}N$ values of *C. variabilis* were significantly higher than those of reference autotrophic plants in Kamuikotan, Aso (individuals with no mycorhizomes formation) and Nagano (Table S5; $P < 0.05$). A significant difference in $\delta^{15}N$ values was found between *C. variabilis* and saprobic fungi at Aso, but the difference was not estimated at the sites of Kamuikotan and Nagano (Fig. 3, Table S5; $P > 0.05$). Although no statistical test was applicable, *C. variabilis* individuals at Saji had $\delta^{15}N$ values close to those of three saprobic fungi, but far from two Ericaceae mycoheterotrophic plants (Fig. 3, Table S5). Several putative ectomycorrhizal OTUs, such as Atheliaceae, Sebacinaceae, Tricholomataceae, *Lactarius* sp. and *Tomentella* sp. were also detected from *C. variabilis* collected from the six sites (Tables S2, S3). AS-4 with no ectomycorrhizal mycobiont shows higher $\delta^{15}N$ values than that of AS-2 with ectomycorrhizal mycobiont (Sebacinaceae phylotype D) (Table S2, Fig. 3). Same correlation was also confirmed between NA-6 with no ectomycorrhizal mycobiont and NA-1, NA-3 with Sebacinaceae phylotype D (Table S2, Fig. 3). High $\delta^{15}N$ values variations were found among *C.variabilis* individuals with various mycobionts collected from ectomycorrhizal/arbuscular

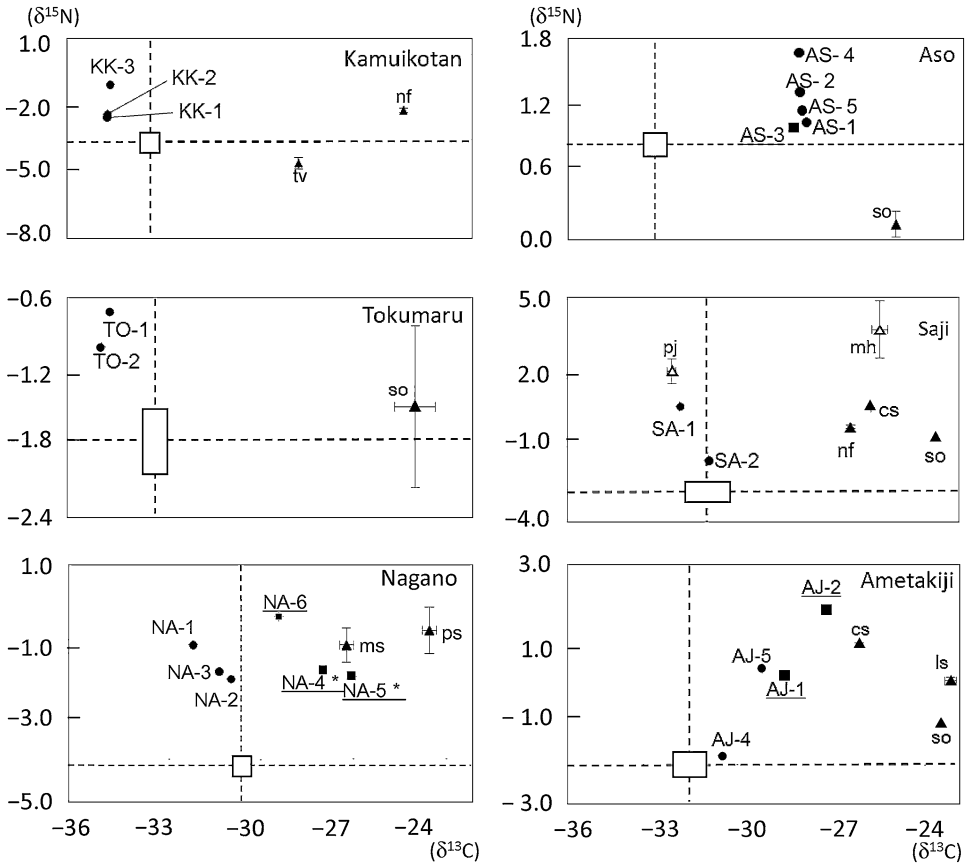

Fig. 3 $\delta^{15}N$ and $\delta^{13}C$ values of *Cremastra variabilis* plants, C_3 autotrophic plants, known mixotrophic species and mycoheterotrophic species, together with fruit bodies of saprobic fungi, at six Japanese sites. Black circles: *C. variabilis* without mycorhizome; black squares: *C. variabilis* with mycorhizome (individual codes are shown close to each symbol and individuals codes with mycorhizome formation are underlined). Attached asterisk with the code shows individuals with branched mycorhizome. Black triangles: means of fruit bodies ± SD in saprobic fungi (nf: *Nematoloma fasciculare* (Hudson: Fr.) Kummer $n = 12$; TV: *Trametes versicolor* (L.) Lloyd $n = 5$; so: *Strobilurus ohshimae* (Hongo et Matsuda) Hongo et Izawa. $n = 13$; ps: *Pluteus* sp. $N = 5$; ms: *Mollisia* sp. $N = 5$; cs *Coprinellus* sp. $N = 10$; ls: *Lepista sordida* (Schumach.) Singer $n = 5$; reference saprobic fruit bodies, $n = 55$). White triangles: means of MX or mycoheterotrophic species ± SD (pj: *Pyrola japonica* Alef.; mh: *Monotropastrum humile* (D.Don) Hara; reference mycoheterotrophic plants, $n = 10$). The white boxes represent mean stable isotope values ± SD for the C_3 reference plants that were sampled together with *C. variabilis* at each site ($n = 140$) and the dotted lines indicate lines of average $\delta^{15}N$ and $\delta^{13}C$ for autotrophs. Twenty-four C_3 plant species used as reference plants for 5 replications, and *Adenocaulon himalaicum* Edgew., *Iris japonica* Thunb., *Polystichum tripteron* (Kunze) C.Presl, Epimel. and *Zingiber mioga* Roscoe were collected twice from two different sites, respectively (see: Table S1)

and arbuscular forest, but differences in $\delta^{15}N$ value in *C. variabilis* due to forest types were not confirmed.

Discussion

Morphology and mycorrhizal symbiosis in *C. variabilis*

Cremastra species display four types of underground organ. The chlorophyllous *Cremastra unguiculata* Finet. has long subterranean stems and forms corms with roots at the end of these organs (Maekawa 1971; Nakajima 2012). The achlorophyllous *Cremastra aphylla* T. Yukawa. develops mycorhizomes and short roots (Yagame et al. 2018; Yukawa 1999). In this study, most chlorophyllous *C. variabilis* individuals formed corms and roots, but some also developed mycorhizomes with corms. *Cremastra variabilis* thus has the ability to form both organs usually found in chlorophyllous orchids (roots) and achlorophyllous orchid (mycorhizomes) of its genus.

From phylogenetic analyses of mycobionts, ecological characteristics of mycobionts and intermediate mycorrhizal symbiosis between chlorophyllous and achlorophyllous orchid in *C. variabilis* were revealed. In addition, influences of specific fungal OTUs onto ^{13}C enhancement and mycorhizome development were also detected. We detected 24 OTUs in Tulasnellaceae, Ceratobasidiaceae and Sebacinales, as well as 5 OTUs in Psathyrellaceae in *C. variabilis*. The three former fungal groups (rhizoctonias) are known as

main mycobionts in chlorophyllous orchids (e.g. Dearnaley et al. 2013b; Jacquemyn et al. 2015; Pecoraro et al. 2010; Shefferson et al. 2010; Těšitelová et al. 2013). In addition, Sebacinaceae are also known as common endophytes and symbionts in achlorophyllous orchids (Weiss et al. 2016), whereas Psathyrellaceae were detected on several mycoheterotrophic orchids (Martos et al. 2009; Ogura-Tsujita and Yukawa 2008; Yamato et al. 2005), with several independent Psathyrellaceae clades recruited in such associations (Selosse et al. 2010). Since in addition Psathyrellaceae supports mycoheterotrophic germination of *C. variabilis* seeds (Yagame et al. 2013), we regard them as mycobionts of *C. variabilis*, together with rhizoctonias (Dearnaley et al. 2013b). In a previous study, the ability of pathogenic fungi for seed germination in the chlorophyllous orchid, *Spiranthes sinensis* (Pers.) Ames. was confirmed (Masuhara and Katsuya 1994). Pope and Carter (2001) also discovered that an Australian chlorophyllous orchid (*Pterostylis* sp.) associates with Ceratobasidiaceae related to *Rhizoctonia solani* J.G. Kühn (=*Thanatephorus cucumeris* (Frank) Donk) and the mycobiont is closely related to the pathogenic fungi. *Cremastra variabilis* may thus associate with Ceratobasidiaceae fungi related to pathogens. Meanwhile, fungi belonging *Leptodontidium*, *Tomentella, Lactarius, Crinipellis* and Atheliaceae were also detected on *C. variabilis* (Table S3). These fungal taxa are also detected on the orchids *Platanthera hyperborea* (L.) Lindl., *Cephalanthera* spp., *Lecanorchis* spp. and *Gastrodia nipponica* Tuyama. (Dearnaley et al. 2013a, b), and they may be mycobionts or endophytes in chlorophyllous and achlorophyllous orchids. All six OTUs in Sebacinaceae, phylotypes A and B in Serendipitaceae, Atheliaceae, *Tomentella* and *Lactarius* could be also ectomycorrhizal. Phylotypes A and B in Serendipitaceae were related to ectomycorrhizal fungi on *Dipterocarpus* sp. (AB854712; Kaewgrajang et al. 2014): indeed, some Serendipitaceae are ectomycorrhizal, although the vast majority of them are mycorrhizal mycobionts in Ericaceae and chlorophyllous orchids (Weiss et al. 2004; 2016). For mycobionts in *C. variabilis*, the proportion of ectomycorrhizal fungi reached 12.5% (53/422), whereas saprobic and potential plant pathogenic fungal OTUs reached 87.4% (369/422) in total. Therefore, the ecological characteristics of main mycobionts in *C. variabilis* could be saprobic fungi.

In general, orchids show specificity for fungal partners (Dearnaley et al. 2013a, b), but several species with main mycorrhizal fungi associate with various fungal partners, such as *Erythrorchis altissima* (Blume) Blume, *Aphyllorchis montana* Rchb.f., *Orchis* spp. etc. (Jacquemyn et al. 2010; Ogura-Tsujita et al. 2018; Roy et al. 2009a, b). Mycorrhizal symbiosis of the chlorophyllous orchid *Oeceoclades maculata* (Lindl.) Lindl. (a tropical African species naturalized in America; Stern 1988) is similar to that of *C. variabilis* in having as main mycobiont *P. candolleana*, whose symbiosis with this orchid was confirmed at the germination stage (Bayman et al. 2016). Moreover, typical orchid mycobionts, *Ceratobasidium* and *Tulasnella*, were also detected as mycobionts on *O. maculata* roots (Bayman et al. 2016). Since both *C. variabilis* and *O. maculata* have huge distribution areas, they may adapt to various environmental conditions. They can grow in various shaded forests with ectomycorrhizal and/or arbuscular mycorrhizal trees. Thus, in addition to rhizoctonias at the adult stages, they may flexibly associate with ectomycorrhizal or saprobic fungi, thereby increasing opportunities to gain nutrients from fungal partners. This ability may allow colonization of various environmental conditions, and this may be validated in *O. maculata*.

Five Psathyrellaceae OTUs were detected on *C. variabilis* in the present study (Table S2). Phylotype E was identified as *Co. domesticus*, a fungus promoting in vitro seed germination and mycorhizome formation in *C. variabilis* (Yagame et al. 2013). In orchids, some mycoheterotrophic species form conspicuous mycorhizomes belowground. The genus *Corallorhiza* comprises 11 mycoheterotrophic species that do not form roots but develop mycorhizomes (Freudenstein 1997), a morphological feature also found in mycoheterotrophic species such as *Chamaegastrodia sikokiana* Makino & F. Maek., *Yoania japonica* Maxim., *Cymbidium macrorhizon* Lindl., *Epipogium aphyllum* Sw., etc. (Maekawa 1971; Roy et al. 2009a, b). Branched mycorhizome formation was also found in chlorophyllous orchid, *Oreorchis indica* (Lindl.) Hook.f. which is closely related species to *Corallorhiza* and *Cremastra*. Mixotrophic feature of this orchid associating with ectomycorrhizal *Tomentella* was confirmed, from which shift from autotrophy to mycoheterotrophy was supported (Suetsugu et al. 2021). *Cremastra variabilis* may increase the ability to gain nutrients from Psathyrellaceae mycobionts with development of mycorhizomes. In the present study, Psathyrellaceae fungi were always detected on *C. variabilis* mycorhizomes. Psathyrellaceae phylotype E were detected from branched mycorhizome, whereas phylotype B detected from non-branched mycorhizome. Interestingly, the individuals associated with Psathyrellaceae phylotype B and E formed mycorhizome, whereas individuals exclusively associating with rhizoctonias did not develop mycorhizome. In further studies, morphological differences in *C. variabilis* associating with rhizoctonias and Psathyrellaceae should be tested in symbiotic culture conditions.

Mycoheterotrophy in *C. variabilis*

The present study supports MX in *C. variabilis*. *Cremastra variabilis* could change ^{13}C relative abundance in their leaves due to fungal partners. In fact, the individuals NA-4, NA-5 and NA-6 with Psathyrellaceae phylotype E (Table S2) showed significantly high relative abundance of ^{13}C compared to reference autotrophic plants (Table S4).

Four individuals collected at the Aso site showed relatively high relative abundance of ^{13}C. Psathyrellaceae phylotype B (main Psathyrellaceae mycobiont in Aso site; Table S2) could enhance the relative abundance of ^{13}C in *C. variabilis*. This phylotype was also detected on AJ-1 and AJ-2, in which AJ-2 shows high relative abundance of ^{13}C. The individuals associated with rhizoctonias showed relatively low $\delta^{13}C$ values that did not differ from those of C_3 autotrophic plants in Kamuikotan and Nagano (Tables S2, S4; Fig. 3). Previous studies reported that $\delta^{13}C$ values did not differ between C_3 plants and leaves of adult rhizoctonia-associated orchids (e.g., Selosse and Martos 2014; Stöckel et al. 2014). Low relative abundance of ^{13}C could even result from association with rhizoctonias (Selosse and Martos 2014; Table S2). However, $\delta^{15}N$ values in *C. variabilis* were higher than those of C_3 plant references so that rhizoctonia-associated *C. variabilis* could be 'cryptic mycoheterotrophs' (sensu Hynson et al. 2013). These results revealed that the combination and specificity of mycobionts could affect the relative abundances of ^{13}C and ^{15}N in *C. variabilis*. For comparison between *C. variabilis* and saprobic fungi in $\delta^{15}N$ values, significant differences were found in Aso, but not in Nagano and Kamuikotan. Since different fungal references were applied for the analyses in $\delta^{15}N$ values in the three sites, fungal physiological differences could affect the differences in $\delta^{15}N$ values among *C. variabilis* and saprobic fungi.

The orchid has newly found MX features supported by various saprobic fungi including non-rhizoctonias. In further studies, MX nutrition in *C. variabilis* associated with various fungi should be tested in symbiotic culture conditions to reveal the influence of each mycobiont on the relative abundances of ^{13}C and ^{15}N.

Conclusion

Cremastra variabilis associates with various fungi, among which the main mycobionts were rhizoctonias (main mycobionts of chlorophyllous orchids) and Psathyrellaceae (hitherto mostly detected on mycoheterotrophic orchids). Psathyrellaceae were constantly found in *C. variabilis* individuals with mycorhizomes, whereas rhizoctonias were mainly found in individuals without mycorhizomes. High variations in stable isotope content among individuals were found, in particular for the relative abundance of ^{13}C which is exceptionally variable in this species. Combination of mycobionts could affect the relative abundances of ^{13}C and ^{15}N in *C. variabilis*. Mixotrophy in *C. variabilis* harboring non-rhizoctonia saprobic fungi was confirmed, which is new to science. Specific Psathyrellaceae phylotypes could affect abundance of ^{13}C and development of branched mycorhizome, whereas the influence of rhizoctonias on the abundance of ^{15}N was also inferred. *Cremastra variabilis* is distributed over a huge area from Sakhalin Island in Russia, to the Himalayas, and this flexibility may help adaptation to various environmental conditions. The orchid could be a suitable model species for comparison of the physiological characteristics of symbiotic cultures using different mycobionts. Because we can easily culture mycobionts of this orchid and resynthesize symbiotic cultures under artificial conditions (Yagame et al. 2013), *C. variabilis* is also an interesting model for the study of the evolution of mycoheterotrophy.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10265-021-01337-w.

Acknowledgements We thank N. Sakata, M. Matsui, K. Hashiba-Kodaira, Y. Katayama, N. Nitta, H. Yoshinaka, and H. Sato, for help with sampling and H. Abe and Y. Yamashita for support with technical assistance for obtaining molecular data. Some analyses were performed at SSMIM (Muséum National d'Histoire Naturelle, Paris) with technical assistance from Denis Fiorillo (UMR 7209 CNRS). This work was supported by a research grant from the National Museum of Nature and Science, The Global COE Program "Advanced Utilization of Fungus/Mushroom Resource for Sustainable Society In Harmony with Nature (15K14442)," and JSPS KAKENHI Grant Numbers (15H04417).

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