The giant mycoheterotrophic orchid *Erythrorchis altissima* is associated mainly with a divergent set of wood-decaying fungi

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Running title: Mycorrhizal generalist with wood-decay fungi

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Abstract

The climbing orchid *Erythrorchis altissima* is the largest mycoheterotroph in the world. Although previous *in vitro* work suggests that *E. altissima* has a unique symbiosis with wood-decaying fungi, little is known about how this giant orchid meets its carbon and nutrient demands exclusively via mycorrhizal fungi. In this study, the mycorrhizal fungi of *E. altissima* were molecularly identified using root samples from 26 individuals. Furthermore, *in vitro* symbiotic germination with five fungi and stable isotope compositions in five *E. altissima* at one site were examined. In total, 37 fungal operational taxonomic units (OTUs) belonging to nine orders in Basidiomycota were identified from the orchid roots. Most of the fungal OTUs were wood-decaying fungi, but underground roots had ectomycorrhizal *Russula*. Two fungal isolates from mycorrhizal roots induced seed germination and subsequent seedling development *in vitro*. Measurement of carbon and nitrogen stable isotope abundances revealed that *E. altissima* is a full mycoheterotroph whose carbon originates mainly from wood-decaying fungi. All of the results show that *E. altissima* is associated with a wide range of wood- and soil-inhabiting fungi, the majority of which are wood-decaying taxa. This generalist association enables *E. altissima* to access a large carbon pool in woody debris and has been key to the evolution of such a large mycoheterotroph.

Keywords

mycoheterotrophy, mycorrhiza, orchid, stable isotope, symbiotic germination, wood-decaying fungi

Introduction

Mycorrhizas are an ancient, widespread association between fungi and land plants. They are based on a mutualistic symbiosis in which the fungus provides water and nutrients to the plant in return for fixed carbon from the plant (Smith & Read, 2008). Although these mutualistic associations are widespread among the majority of photosynthetic plants, mycoheterotrophic (MH) plants, which have evolved independently in 17 plant families (Merckx et al., 2013), have completely lost their photosynthetic ability and obtain all of their carbon through mycorrhizal associations (Leake, 1994). In most cases, MH plants rely on the two dominant mycorrhizal symbioses, the arbuscular mycorrhizal association and ectomycorrhizal (ECM) association, which allow MH plants to obtain carbon from surrounding autotrophic plants via shared mycorrhizal mycelia (Merckx, 2013). Whereas such tripartite systems provide access to the common mycorrhizal network of arbuscular mycorrhizal and ECM fungi linking the autotrophic plants (Bidartondo, 2005), associations with free-living litter- or wood-decaying (WD) fungi have been shown in several MH orchids. Early studies based on the isolation technique found this association in several MH orchids, such as *Gastrodia elata* (Kusano, 1911) and *Cyrtosia septentrionalis* (as *Galeola septentrionalis*) (Hamada, 1939) associating with the plant pathogenic WD fungus *Armillaria*, *Gastrodia javanica* associating with the WD polypore *Xerotus javanicus*, and *Didymoplexis minor* associating with the litter-decaying fungus *Marasmius coniatus* (Burgeff, 1932). Recent molecular work has also confirmed the association of tropical or warm-temperate MH orchids with WD fungal linages, such as *Epipogium roseum* with Psathyrellaceae (Yamato et al., 2005), *Eulophia zollingeri* with *Psathyrella candolleana* (Ogura-Tsujita & Yukawa, 2008), *Gastrodia similis* with *Resinicium*

(Martos et al., 2009), and *Cyrtosia* and *Galeola* species with Meripilaceae (Umata et al., 2013; Lee et al., 2015). Furthermore, litter-decaying Mycenaceae and Marasmiaceae have been found to associate with MH orchids, such as *Wullschlaegelia aphylla* (Martos et al., 2009) and *Gastrodia* species (Ogura-Tsujita et al., 2009; Lee et al., 2015; Kinoshita et al., 2016; see Selosse et al., 2010 for more detail). Decomposition of woody debris and leaf litter by saprotrophic fungi plays a key role in regulating the carbon (C) and nutrient cycles of all terrestrial ecosystems (Berg & McClaugherty, 2003). Woody debris is a major component of forest biomass, and this large C store represents up to 20% of the total aboveground biomass (Laiho & Prescott, 1999; Bradford et al., 2009). MH plants that are associated with saprotrophic fungi likely depend on the forest C cycle from plant debris, but understanding of mycorrhizal associations with litter- or wood-decaying fungi is still limited.

The giant mycoheterotroph *Erythrorchis altissima* (Blume) Blume (as *Galeola altissima* and *Erythrorchis ochobiensis*) is expected to have a unique symbiosis with WD fungi, which could act as a new model for understanding mycorrhizal diversity and specificity in MH plants. This species is the largest mycoheterotroph. It is a climbing, perennial hemi-epiphytic orchid species without foliage leaves, with both an aerial and subterranean root system, and with a distribution ranging from warm-temperate to tropical regions in East to South East Asia (Comber, 1990; Figure 1). Its stems climb over dead wood or living trees, and often reach a length of 10 m (Averyanov, 2011). Despite such remarkable characteristics of *E. altissima*, the fundamental basis of how it meets its C and nutrient demands exclusively via mycorrhizal fungi is unknown. Early research by Hamada and Nakamura (1963) and previous *in vitro* studies (Umata, 1995, 1997a, b, 1998a, b, 1999; Umata et al., 2000; see

more details in Table S1) have shown that 19 basidiomycete species, most of them WD fungi that were never previously shown to be mycorrhizal fungi, had mycorrhizal association with *E. altissima*. These studies indicate that *E. altissima* is a mycorrhizal generalist, targeting a wide phylogenetic range of WD basidiomycetes, which has not been demonstrated for any other plant.

An association with ECM fungi has also been suggested, as shown by successful germination with the ECM fungus *Lyophyllum shimeji* (Umata, 1997b). In fact, both saprotrophic *Gymnopus* and the ECM fungus *Russula* have been identified from underground roots in *Erythrorchis cassythoides* (Dearnaley, 2006), which is the sister species of *E. altissima* and is also a climbing mycoheterotrophic orchid in Australia (Jones, 2006). Based on these studies, *E. altissima* is assumed to lack fungal specificity, targeting a range of wood-inhabiting fungi in addition to ECM fungal associations, which indicates a mixed C gain from WD and ECM fungi. Stable isotope natural abundance can be used to assess a plant's nutritional mode and is particularly useful in MH plants that fully depend on fungal-derived C and nitrogen (N) as they are heavily enriched in ^{13}C and ^{15}N (Gebauer & Meyer, 2003). This approach has been applied to a number of MH species associated with ECM fungi (Bidartondo et al., 2004; Abadie et al., 2006; Liebel et al., 2010), arbuscular mycorrhizal fungi (Merckx et al., 2010; Bolin et al., 2015) and also saprotrophic fungi (Martos et al., 2009; Ogura-Tsujita et al., 2009; Lee et al., 2015). The difference in isotopic signatures between WD and ECM fungi can distinguish which fungal group covers the majority of the C and N demand of *E. altissima* (Kohzu et al., 1999; Hobbie et al., 2012).

123 This study is the first to investigate the mycoheterotrophy of *E. altissima*
124 comprehensively by combining molecular, *in vitro* culture and mass-spectrometric approaches.
125 To reveal its mycorrhizal fungal diversity and specificity, we first analyzed 26 individuals
126 from six sites using molecular identification. Second, to confirm the mycorrhizal potential of
127 identified fungi, we isolated five mycorrhizal fungal strains from root tissues and used them
128 for co-culture with seeds in conjunction with a decay test to compare the wood-decay ability
129 of these isolates. Third, natural stable isotope abundances of C and N were analyzed to
130 confirm the mycoheterotrophy and reveal the pathways for nutrient acquisition in *E.*
131 *altissima*.
132
133 **Materials and Methods**
134
135 Field sites and sample collection
136
137 Plant and fungal materials were collected from six sites of warm-temperate (S1–S3) or
138 subtropical (S4–S6) regions in Japan from 2013 to 2016 (Table 1, Figure S1). The habitats of
139 *E. altissima* were shaded to semi-open places in evergreen broadleaf forests dominated by
140 *Castanopsis sieboldii*. Most of the individuals found in this study were hemi-epiphytes with
141 stems climbing on fallen or standing dead trunks and living trees from underground (Figure
142 1a, b); however, a few individuals were creeping on the ground without host trees. The
143 average length of aboveground stems among 29 individuals was 3.9 m, ranging from 1.5 to
144 7.0 m at site S6. The most common host tree species was *C. sieboldii* at all sites, but

145 *Distylium racemosum*, *Elaeocarpus japonicus*, *Elaeocarpus zollingeri*, *Myrsine seguinii*,
146 *Syzygium buxifolium*, and *Cinnamomum daphnoides* were also found (Tables 2, 3). The level
147 of decay of host trees was surveyed according to Fukasawa et al. (2009) and assigned to five
148 classes: 1) wood, hard; 2) wood, somewhat hard, a knife penetrates less than 1 cm into the
149 wood; 3) wood, distinctly softened, a knife penetrates ~1–4 cm into the wood, bark partly
150 lost; 4) wood, strongly decayed, a knife penetrates ~5–10 cm into the wood, bark lost in most
151 places; and 5) wood, very decayed, a knife penetrates more than 10 cm into the wood, original
152 log circumference not recognizable or hardly recognizable.
153 Root morphology was categorized into two groups: thick and densely branched root
154 clumps (Figure 1c, e) and thin and elongate roots (Figure 1d). Both types appeared in aerial
155 (Figure 1c, d) and underground (Figure 1e) plant stems. Mycorrhizal colonization was
156 confirmed with a light microscope using free-hand sections of all collected roots. Our
157 preliminary observation showed that mycorrhizal fungi mainly colonized densely branched
158 roots (Figure 2) while elongate roots were scarcely colonized. Thus, the former roots were
159 used mainly for the following microscopy observations and molecular identification.
160 As mycorrhizal association with WD fungi has been suggested by previous studies
161 (Hamada & Nakamura, 1963; Umata, 1995, 1997a, b, 1998a, b, 1999; Umata et al., 2000),
162 sporocarps of WD fungi were also collected from host trees of *E. altissima* and identified at
163 the species level by morphology or molecular identification. Voucher specimens of *E.*
164 *altissima* and sporocarps were deposited in the Herbarium of the National Museum of Nature
165 and Science, Tokyo (TNS8501221, 8505147, 8505854–8505857 for *E. altissima*, and
166 TNS-F-80541, 80542 for *Trichaptum* cf. *durum*) and in the Tottori University Mycological

Herbarium (TUMH62765 for *Coniophorafomes matsuzawae*).

Microscopy observation

For assessment of mycorrhizal colonization in root tissues, collected mycorrhizal roots were fixed in 50% ethanol/formaldehyde/acetic acid, 90:5:5 for microscopy observation. Root pieces were dehydrated in a graded ethanol series, embedded in paraffin, cut transversely into 10-μm-thick sections, and stained with safranin-O/fast green. The sections were dehydrated through an alcohol-xylene series, mounted with Bioleit (Oken Shoji, Tokyo, Japan), and fungal colonization was observed under a light microscope.

Molecular identification of mycorrhizal fungi

In total, 150 roots from 26 individuals were collected from six sites for molecular identification of mycorrhizal fungi (Table 1). One to 14 root pieces were collected from each individual, and when the individuals had several root clumps on the host tree, root tips were collected from each clump because our preliminary observation showed that if there are several independent rooting zones, each root clump establishes mycorrhizas separately. To check the annual change in mycorrhizal associations, the roots were collected each year from the same individual (individuals Ea3 and Ea4) for 3 years (Table 2). Collected roots were washed in water and sectioned with a razor blade, and fungal colonization was confirmed with a light microscope. To avoid detection of surface-inhabiting non-mycorrhizal fungi, the

189 root epidermis was removed from mycorrhizal root tissues and the colonized cortex layer was
190 excised under a stereomicroscope. For sporocarps, a piece of tissue was excised from collected
191 sporocarps and used for molecular identification. The excised mycorrhizal roots and
192 sporocarps were washed in sterilized water and stored in TE buffer (10 mM Tris, 1 mM
193 EDTA, pH 7.5) at −20°C before use.
194 DNA was extracted from the samples of mycorrhizal roots and sporocarps using a
195 DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol.
196 PCR and sequencing were performed as described by Ogura-Tsujita and Yukawa (2008). The
197 fungal internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) was
198 amplified with ITS1F/ITS4 or ITS1F/ITS4B primer combinations (White et al., 1990; Gardes
199 & Bruns, 1993). To avoid overlooking Tulasnellaceae, a typical orchid symbiont, due to primer
200 mismatch, all root samples were also amplified using the ITS1/ITS4-Tul primer combination
201 (Taylor & McCormick, 2008). The partial large subunit (LSU) nrDNA sequences were
202 additionally amplified using LR0R/LR5 primers (Moncalvo et al., 2000) when the ITS
203 sequence had low resolution in a homology search of the GenBank database. Additional
204 internal primers, ITS2 and ITS3 (White et al., 1990) for the ITS region and LR3 (Vilgalys &
205 Hester, 1990) and LR3R (Hopple & Vilgalys, 1999) for the LSU region were used for
206 sequencing. The PCR products were purified using a Fast Gene Gel/PCR Extraction Kit
207 (Nippon Genetics, Tokyo, Japan) and sequenced using a BigDye Terminator v3.1 Cycle
208 Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA). PCR products that were
209 difficult to sequence directly were cloned using a pGEM-T Vector System II (Promega,
210 Madison, WI, USA). Five colonies were sequenced in each cloned sample. Obtained sequences

were grouped into operational taxonomic units (OTUs) at 99% similarity, and taxonomic affiliations for each fungal OTU were assigned based on the closest match to sequences available in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences determined in this study were deposited in the DDBJ/EMBL/GenBank databases. The accession numbers are listed in Table S2 and Table S3.

Symbiotic germination

To test whether the mycorrhizal fungi identified in this study induce symbiotic germination of *E. altissima*, mycorrhizal fungi were isolated from roots collected at site S1 in 2013 by the single peloton isolation method (Rasmussen, 1995). Colonized cortex layers of mycorrhizal roots were excised under a stereomicroscope, rinsed three times with sterile water, and cut open under sterile water to release the fungal pelotons. Sterile water mixed with pelotons was dropped onto 2% malt extract agar (MA) plates and incubated at 25°C in the dark. After three days, fungal hyphae growth from coiled pelotons was checked under a light microscope and hyphal tips were transferred to fresh MA plates for subculture and purification. DNA was extracted from fungal isolates as described by Izumitsu et al. (2012) and fungal OTUs were molecularly identified. In total, five fungal isolates that shared 100% ITS sequence homology with the mycorrhizal fungi directly sequenced from colonized roots were used for the co-culture of seeds (Table 4). These isolates were deposited in NITE Biological Resource Center (NBRC110364–110370; Table 4).

A mature fruit of *E. altissima* was collected from site S1 in October 2013. Seeds were stored at 5°C with silica gel until use. Co-culture of seeds and fungi was performed as described by Umata (1997a). Sawdust medium containing 80 mL of *Fagus crenata* sawdust and 40 mL of culture solution (water, 1% glucose, 1% yeast powder) was prepared in a 200-mL conical flask and autoclaved twice at 98°C for 2 h followed by 210°C for 1 h. The seeds were sterilized with a 10% calcium hypochlorite solution as described by Umata (1997a) and ~100 seeds were sprinkled in a sterilized bamboo stick. Each seed stick was incubated for 2 weeks on potato dextrose agar medium to check for contamination of the seeds, and contaminated sticks were removed. Four seed sticks were transferred to sawdust medium and four flasks were prepared for each fungal isolate. A 3 × 3-mm^2 block of fungal culture was inoculated on the surface of the sawdust medium and cultured for 2 months at 25°C in the dark. The experiment was repeated three times with four flasks per replicate and in total 12 flasks were prepared for each isolate. Seed germination was recorded 2 months after sowing and assigned to two germination stages: stage 1 involved rupture of the testa by the enlarged embryo and included protocorms less than 3 mm in diameter; stage 2 included non-rooted protocorms above 3 mm in diameter or rooted protocorms (Figure 3a). For further development under symbiotic condition, obtained seedlings by culturing with the two isolates (T-13 and T-36) that induced seed germination were transferred to fresh sawdust medium (Figure 3b). As the fungal isolates were colonized in seedling roots, the isolates were also transferred to the medium together with the seedlings. Mycorrhizal roots were collected from a plantlet and colonizing fungus was molecularly identified to confirm whether the root-colonizing fungus in a plantlet was consistent with the original isolates.

254

255 Decay test

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257 It seems likely that a WD fungus with strong decay ability may supply carbon stably to the
258 orchid and *E. altissima* could prefer such fungus. To evaluate how the fungal decay ability
259 affects orchid seed germination, five isolates used for co-culture were employed for
260 comparison of wood-decay ability based on sawdust weight loss. Approximately 1 g of
261 oven-dried sawdust from *C. sieboldii*, which is a common *E. altissima* host tree, was packed in
262 a mesh bag and weighed prior to fungal inoculation. The bags were autoclaved at 121°C for 20
263 min and transferred to plates containing 20 mL of 2% agar medium. A 4-mm plug of fungal
264 culture was inoculated on the agar plates and incubated at 25°C in the dark. After 5 months of
265 culture, the bags were oven-dried at 70°C for 1 week and weighed. The weight lost from the
266 sawdust was determined as a percentage of the initial mass. Three replicates were prepared in
267 each isolate, and three non-inoculated plates served as a control.

268

269 Isotopic analysis

270

271 Plant and fungal samples for stable isotope natural abundance analysis were collected at site S1
272 in July 2015. Flower stalk (peduncle and rachis), flower, mycorrhizal and/or non-mycorrhizal
273 root(s) were sampled from five individuals of *E. altissima* (individual IDs Ea3, Ea4, Ea10,
274 D113, and D114; Figure 4, Table S4) which were all flowering individuals in this site. The
275 individuals labeled Ea3 and Ea4 grew on fallen dead trunks of *D. racemosum* while the other

276 three individuals grew on standing dead trunks or living trees of *C. sieboldii* whose heartwood
277 and main branches were partially decayed. Mycorrhizal roots for molecular identification were
278 collected from these individuals (Table 2) except for one individual (D114) that had no root
279 clump aboveground. Collection of underground roots from any of the five individuals would
280 have required major disturbances and was avoided for conservation reasons. Current-year
281 leaves and stems of autotrophic reference plants, *C. sieboldii*, *D. racemosum*, *Psychotria*
282 *serpens*, *Damnacanthus indicus*, and *M. seguinii*, were collected within 1 m of each orchid
283 individual (Table S4). Dead stem-wood material, which was expected to be the main substrate
284 for WD fungi, was sampled from each host tree. In total, five sporocarps, *T.* cf. *durum* from host
285 trees of Ea3 and Ea4, a WD fungus *Microporus* sp. from neighboring *C. sieboldii* and ECM
286 *Amanita* and *Ramaria* species within 10 m of *E. altissima* individuals, were also collected. All
287 sporocarps were identified by morphology or molecular identification and deposited as dried
288 herbarium specimens (TNS-F-80541–80544, 80568). Samples were dried at 105°C, ground to a
289 fine powder and stored in a desiccator with silica gel until use.
290 The relative N and C isotope abundances of the samples were measured using the
291 dual-element analysis mode of an elemental analyzer coupled to a continuous flow isotope ratio
292 mass spectrometer as described in Bidartondo et al. (2004). Relative isotope abundances are
293 denoted as δ values, which were calculated according to the following equation: $\delta^{15}N$ or $\delta^{13}C$ =
294 $(R_{sample}/R_{standard} - 1) \times 1000‰$, where R_{sample} and $R_{standard}$ are the ratios of heavy isotope to light
295 isotope in the samples and the respective standard. Standard gases (nitrogen and carbon
296 dioxide) were calibrated with respect to international standards using the reference substances

297 N1 and N2 for N isotopes and ANU sucrose and NBS 19 for C isotopes, provided by the
298 International Atomic Energy Agency (Vienna, Austria).
299 δ values were normalized following the procedure of Preiss and Gebauer (2008) for
300 our comparisons of plant C and N isotope abundances with reference data. Enrichment factors
301 ($\varepsilon^{13}C$ and $\varepsilon^{15}N$) were calculated using δ values for *E. altissima*, the reference plants, and
302 sporocarps as follows: $\varepsilon_{Sx} = \delta_{Sx} - \delta_{REFx}$, where *S* is a single $\delta^{13}C$ or $\delta^{15}N$ value for each sample,
303 *x* is a sampling plot within a certain study site, and δ_{REF} is the mean value of all reference plants.
304 Differences between $\varepsilon^{13}C$ and $\varepsilon^{15}N$ values of *E. altissima* and each reference plant, and between
305 the stem and leaf of each reference plant, were determined using a Mann-Whitney *U*-test. A
306 Kruskal-Wallis nonparametric test was used for differences among flower stalks, flowers, and
307 roots of *E. altissima*.
308 Non-metric multidimensional scaling (NMDS) was used to detect meaningful
309 underlying dimensions and to graphically visualize similarities and dissimilarities between the
310 samples of *E. altissima* and WD fungi as well as decayed wood samples collected from *D.*
311 *racerosum* and *C. sieboldii* in two-dimensional space. For this, the Bray-Curtis index was used
312 to calculate a distance matrix from $\varepsilon^{13}C$, $\varepsilon^{15}N$, and N concentration data using the function
313 'metaMDS' with two dimensions and 100 permutations in the R package 'vegan' (Oksanen et
314 al., 2017). The stress value was calculated to evaluate how well the configuration provided a
315 representation of the distance matrix; generally, a stress value of <0.05 provides an excellent
316 representation in reduced dimensions. Fitted vectors were calculated to display the $\varepsilon^{13}C$, $\varepsilon^{15}N$,
317 and N concentrations in the ordination space and to indicate the differences between the groups
318 in association with these variables. Each arrow shows the direction of the increasing response

variable while its length is proportional to the correlation (R^2) between the variable and the ordination (Oksanen et al., 2017). The function 'adonis' in the R package 'vegan' was used to perform a permutational multivariate analysis of variance (MANOVA) to test for significance of differences between group means using the aforementioned calculated distance matrix (Anderson, 2001).

Results

Molecular identification of mycorrhizal fungi

In total, 150 root samples taken from 26 *E. altissima* individuals from six sites were examined using molecular identification, and fungal sequences were successfully obtained from 141 root samples (Table 1). Basidiomycete sequences were grouped into 37 fungal OTUs based on 99% ITS sequence identity, belonging to nine fungal orders (Table S2). The sequences from two fungal OTUs, *Trichaptum* cf. *durum* and *Coniophorafomes matsuzawae*, completely matched those from adjacent sporocarps. Most of the fungal OTUs were WD basidiomycetes, and ECM fungus Russulaceae and orchid mycorrhizal Ceratobasidiaceae, Tulasnellaceae, and Serendipitaceae were additionally identified from the roots (Table S2). Ascomycete lineages, such as *Ilyonectria* and *Trichosporon*, which are hyphal endophytes, were also detected at low frequency (Table S3).

No common fungal OTU was found among the six sites, except that *Phlebia* sp.2 was detected at both warm-temperate site S1 and subtropical site S6 (Table 2, Table 3). The

detected fungal OTUs differed for each individual in most cases, although an identical fungal OTU was detected from different individuals within site S1 (*T.* cf. *durum*, *Ceriporia* sp.1, *Phlebia* sp.2, and *Gymnopus* sp.1) and site S6 (Ceratobasidiaceae sp.1, *Phanerochaete* sp.3, *Phlebia* sp.2, and *Microporus* sp.1). *Erythrorchis altissima* was present at various tree stages, but no correlation was found between the tree stage and the fungal species detected. The WD basidiomycete *T.* cf. *durum* dominated *E. altissima* roots on fallen dead wood of *D. racemosum* and was the most common through all years of the study period. *Erythrorchis altissima* frequently appeared on the tree trunk at decay-class 3. The fungi detected from underground roots belonged to diverse fungal lineages including both WD and ECM basidiomycetes. Simultaneous association with both fungal groups within a single individual was found in two individuals: Y159 and Y161 (Table 2). The underground roots without aboveground host trees were associated with WD fungus *Ceriporia* sp.1 (Y162 and Ea4D; Table 2). This fungal OTU was detected in both aboveground and underground roots (Table 2).

Symbiotic germination and decay test

Five fungal isolates with ITS sequences that were identical to the mycorrhizal fungi directly sequenced from colonized roots were successfully obtained from four individuals at site S1 (Table 4). Two isolates, *T.* cf. *durum* and *Vuilleminia* sp.1, induced seed germination (Figure 3a), and the number of germinated individuals that inoculated *Vuilleminia* sp.1 was significantly higher than *T.* cf. *durum* (Table 4). The seedlings developed into plantlets with

these isolates after being transplanted into fresh medium (Figure 3b). The wood decay ability of the five isolates was compared using the sawdust weight loss. The average weight losses ranged from 4.1% to 43.5%, with the highest weight losses in *Hyphodontia* sp.1 (43.5%) and *T.* cf. *durum* (41.3%), and the lowest in *Ceriporia* sp.1 (4.1%).

Stable isotope abundances

Among five individuals analyzed from site S1, Ea3 and Ea4 grew on fallen dead trunks of *D. racemosum*, whereas the other three individuals (Ea10, EaD113, and EaD114) grew on standing dead trunks or living trees of *C. sieboldii*. The former two individuals were associated mainly with the wood-decaying *T.* cf. *durum*, and the latter were mycorrhizal with several WD fungi, such as *Hypholoma*, *Phlebia*, and *Phanerochaete* (Table 2). No significant differences in $\delta^{13}C$ or $\delta^{15}N$ were found among orchid flower stalks, flowers, and roots (Kruskal-Wallis test, $P = 0.77$ for $\delta^{13}C$ and 0.81 for $\delta^{15}N$), or between leaves and stems of each reference plant species (Mann-Whitney *U*-test, $P < 0.05$), except for $\delta^{15}N$ values of *D. racemosum* (Table S5). The enrichment factor (ε) based on the stems of reference plants (Figure 4) showed a similar pattern to the ε for the leaves (Figure S2). Thus, the $\varepsilon^{13}C$ and $\varepsilon^{15}N$ values based on the stems are shown as the main data because the stem is the organ equivalent to the flower stalk and was the only material collected from all five *E. altissima* individuals (Table S4).

The $\delta^{13}C$ values of *E. altissima* were significantly enriched compared to those of all reference plant species (Mann-Whitney *U*-test, $P < 0.01$; Table S5). Based on the enrichment

385 factors, all individuals of *E. altissima* were highly enriched in ^{13}C compared to the reference
386 plants, but varied extremely in ^{15}N, ranging from 0.38% to 7.12% in $\varepsilon^{15}N$ values (Figure 4a).
387 The individuals growing on *D. racemosum* did not differ from reference plants in ^{15}N ($\varepsilon^{15}N$:
388 0.38% to 1.60%), whereas those growing on *C. sieboldii* were highly enriched ($\varepsilon^{15}N$: 2.69% to
389 7.12%). Furthermore, the enrichment of ^{13}C and ^{15}N in the two former individuals was the
390 closest to those of *T.* cf. *durum* that dominated the mycorrhizal roots of these individuals,
391 while the latter was close to a WD *Microporus* collected from *C. sieboldii* although the
392 individuals EaD113 and EaD114 ($\varepsilon^{15}N$: 4.70% to 7.12%) were more enriched in ^{15}N than
393 Ea10 ($\varepsilon^{15}N$: 2.69% to 3.89%). The ^{13}C and ^{15}N enrichments for dead-wood material were also
394 quite different between the two tree species of *D. racemosum* and *C. sieboldii* (Figure 4a).
395 Ordination of a Bray-Curtis dissimilarity matrix calculated from $\varepsilon^{13}C$, $\varepsilon^{15}N$, and N
396 concentration data of *E. altissima* and WD fungi as well as decayed wood samples collected
397 from *C. sieboldii* and *D. racemos*um (n = 21) with NMDS elucidated a significant segregation
398 of the two groups in the ordination space (Figure 4b), and a MANOVA showed that the group
399 had a significant effect on the ordination ($R^2 = 0.343$, $P = 0.001$). Fitted vectors in the
400 ordination of *E. altissima* collected from *C. sieboldii* and *D. racemosum* were maximally
401 correlated with $\varepsilon^{15}N$ ($R^2 = 0.683$, $P < 0.001$), N concentration ($R^2 = 0.550$, $P < 0.001$) and $\varepsilon^{13}C$
402 ($R^2 = 0.470$, $P = 0.006$). Generally, the stress value of the ordination (stress = 0.02) provided an
403 excellent representation (Figure 4b). Thus, the different C and N isotope compositions and N
404 concentrations of the two host tree species *C. sieboldii* and *D. racemosum* turned out as drivers
405 for the C and N isotope compositions and N concentrations not only of the wood-decay fungi
406 living on these two tree species, but also for the C and N isotope compositions and N

concentrations of the mycoheterotrophic *E. altissima* individuals collected from the two tree species.

Discussion

Mycorrhizal associations

This study provides clear evidence that *E. altissima* is associated with a wide phylogenetic range of fungi inhabiting wood and soil. The fungi detected in this study belong to nine fungal orders, which include different functional guilds, mainly including WD fungi but also ECM and typical orchid mycorrhizal fungi (Table S2), although the fungi occurring at low frequency will need further confirmation. Most of the WD fungi detected from *E. altissima* roots were first found to be mycorrhizal fungi on plant roots in this study, with the exception of the leaf litter or WD fungi *Gymnopus* and *Mycena*, which are associated with several MH orchids, such as *Gastrodia* species (Xu & Guo, 2000; Martos et al., 2009; Kinoshita et al., 2016) and *E. cassythoides* (Dearnaley, 2006). The ECM genus *Russula* was found on underground roots of *E. altissima*, as shown in *E. cassythoides* (Dearnaley, 2006). *Russula* is a common mycorrhizal partner in MH plants, such as temperate orchids, *Corallorhiza* (Taylor & Bruns, 1997, 1999), *Limodorum* (Girlanda et al., 2006), and monotropoid species of Ericaceae (Bidartondo & Bruns, 2001). The *Russula* sequences from *E. altissima* roots share high sequence similarity with those from ECM root tips (Table S2), indicating that some *E. altissima* individuals partially obtain C from ECM fungi. The typical orchid mycorrhizal

fungi, such as Tulasnellaceae, Ceratobasidiaceae, and Serendipitaceae, were also found in *E. altissima* roots. The ITS sequence of Serendipitaceae sp.1 from roots on decayed wood shared 96% homology with that from *E. cassythoides*, indicating that this fungal group works as a mycorrhizal fungus in *Erythrorchis*. A series of previous studies demonstrated that 19 fungal species induced seed germination by co-culture *in vitro* (Table S1), but we could not detect these fungi from *E. altissima* roots, except for *Microporus* sp.1, which shared 99% sequence homology with *Microporus affinis* and was found in two individuals (Table 3). These results suggest that more fungal species could be associated with *E. altissima* than those found in this study. Ascomycete fungi were also detected from *E. altissima* roots (Table S3), but most of them are common root endophytes or plant root pathogens (Chaverri et al., 2011), thus these fungi are probably non-mycorrhizal on *E. altissima* roots.

This study also provides clear evidence of a WD-associated mycoheterotroph that lacks mycorrhizal specificity. Previous studies showed that WD-associated MH orchids have mycorrhizal specificity towards single fungal orders, genera, or even species groups (Yamato et al., 2005; Ogura-Tsujita & Yukawa, 2008), whereas multiple fungal orders including saprotrophic and ECM fungi were detected in *E. cassythoides* (Dearnaley, 2006) and *Gastrodia nipponica* (Kinoshita et al., 2016). A lack of fungal specificity has been shown in some MH plants, such as the ericaceous mycoheterotroph *Pyrola aphylla*, which is associated with a broad range of ECM fungi (Hynson & Bruns, 2009), and species of the MH orchid *Aphyllorchis* with multiple ECM families (Roy et al*.,* 2009). While the generalist association of *P. aphylla* may be an ancestral trait because a partially mycoheterotrophic *Pyrola* is also a generalist (Hynson & Burns, 2009; Tedersoo et al., 2007), it is notable that the lack of fungal

451 specificity in *E. altissima* has probably evolved from a photosynthetic orchid with a
452 specialized mycorrhizal association. One of the photosynthetic relatives of *E. altissima* within
453 Vanilloideae is the climbing orchid genus *Vanilla* (Cameron, 2009), which is associated
454 mainly with a particular fungal lineage of Ceratobasidiaceae and Tulasnellaceae
455 (Porras-Alfaro & Bayman, 2007).

456 The few common fungal OTUs among the six sites indicate that the differences in
457 fungal OTUs associated with *E. altissima* may reflect differences in the local community of
458 WD fungi, which are attributed to climate, vegetation, and other environmental factors,
459 although randomness of fungal occurrence and contingency should also be considered. Host
460 tree species and their decay-class may also affect which fungal OTU associates with *E.*
461 *altissima*. *Erythrorchis altissima* on fallen decayed wood of *D. racemosum* was frequently
462 associated with *T.* cf. *durum* in this study (Table 2). Wood in decay-class 3 was the most
463 common among the dead host trees of *E. altissima* (Tables 2, 3). In early to mid-stages, WD
464 fungal flora, especially corticioids and polypores, are very species rich (Renvall, 1995;
465 Stokland et al., 2012) and WD basidiomycetes are metabolically active in decayed wood
466 (Rajala et al., 2011), which may provide the opportunity for *E. altissima* to find fungal
467 partners.

468 Underground roots have been associated with ECM *Russula*, similar to *E.*
469 *cassythoides* (Dearnaley, 2006), in addition to WD fungal groups (Table 2). The simultaneous
470 association with both fungal groups within a single individual (Y159 and Y161; Table 2)
471 showed mixed C gain from decayed woods and neighboring ECM-associated autotrophs.
472 Such double association was also found in *Gastrodia nipponica*, which has been associated

473 mainly with litter-decomposing Mycenaceae and Marasmiaceae with additional association
474 with Russulaceae (Kinoshita et al., 2016). The WD fungus *Ceriporia* sp.1 was found from the
475 underground roots of the individuals without a host tree (Y162 and Ea4D; Table 2),
476 suggesting that *E. altissima* can survive without an aboveground host tree by utilizing
477 underground woody debris as a nutrient.
478 Annual root sampling from particular individuals revealed that two individuals (Ea3
479 and Ea4) retained the dominant association with the same fungal OTU, *T.* cf. *durum*, for 3
480 years, although other fungal OTUs were partially associated (Table 2). Mycorrhizal roots
481 collected from four to five root clumps within 1.5 m were exclusively associated with *T.* cf.
482 *durum* in both individuals, and sporocarps of *T.* cf. *durum* were abundant on host logs
483 throughout the study period. These results indicate that this fungal OTU was probably a
484 dominant WD species within these host trunks and continuously supplied nutrients to *E.*
485 *altissima* for at least 3 years.
486
487 Symbiotic germination
488
489 Among the five isolates, *T.* cf. *durum* and *Vuilleminia* sp.1 induced seed germination and
490 subsequent plantlet formation (Table 4), showing that these two fungal groups that were
491 isolated from adult plants are efficient for seed germination *in vitro* as well as mycorrhizal
492 association in adulthood. Assessment of decay ability showed that the fungal isolates that
493 were efficient for seed germination do not require a high-decay ability. As the most effective
494 at seed germination, *Vuilleminia* sp.1 showed low weight loss *in vitro* (24.6%), while

495 *Hyphodontia* sp.1, which did not induce germination, had the highest weight loss (43.5%). No
496 seed germination was observed in three fungal isolates, even though *Ceriporia* sp.1 was one
497 of the most frequent fungal OTUs at site S1. It is possible that fungal specificity is higher in
498 the germination stage than in adulthood, but deviation from optimal culture conditions for
499 some fungal isolates could be one of the possibilities for non-induction of seed germination.
500
501 Stable isotope abundance
502
503 *Erythrorchis altissima* had C isotope signatures typical of a fully mycoheterotrophic orchid.
504 The $\varepsilon^{13}C$ values of *E. altissima* ranged from 7.39% to 13.27% with an average of 9.97%,
505 which is similar to the two MH orchids, *Cyrtosia javanica* and *Galeola falconeri*, both of
506 which are closely related to *E. altissima* (Cameron, 2009) and are also associated with WD
507 Polyporales (11.20 ± 0.68% and 11.87 ± 0.56%, respectively; Lee et al*.,* 2015) and
508 ECM-associated orchids reviewed by Hynson et al. (2016) including 13 MH orchid species
509 (from 6.58 ± 0.24% to 10.78 ± 0.62%). In addition to ^{13}C enrichment, *E. altissima* was highly
510 variable in its ^{15}N enrichment, ranging from 0.38% to 7.12% in the $\varepsilon^{15}N$ values, which is
511 likely due to the difference in host tree species and/or mycorrhizal fungi (Figure 4). An
512 ordination of a Bray-Curtis dissimilarity matrix calculated from $\varepsilon^{13}C$, $\varepsilon^{15}N$, and N
513 concentration data supports the conclusion that the host tree species may affect ^{13}C and ^{15}N
514 enrichment of *E. altissima*, WD fungi, and decayed wood, and might be responsible for the
515 significantly segregated groups.

Although different functional guilds of fungi were associated with *E. altissima*, the comparison of ^{13}C and ^{15}N enrichments with fungal sporocarps showed that *E. altissima* gains C mainly from WD fungi of its host tree. ^{13}C and ^{15}N enrichment of two individuals on *D. racemosum* were similar to the WD fungus *T.* cf. *durum*, which was the main fungal partner of these individuals (Figure 4, Table 2). The enrichments of other individuals on *C. sieboldii* were close to the WD fungus *Microporus* that was collected from *C. sieboldii*. The individuals, EaD114 and EaD113, were more enriched in ^{15}N and seemed to have intermediate values between *Microporus* and ECM *Amanita*. Because ECM-associated mycoheterotrophs are highly enriched in ^{15}N due to high ^{15}N enrichment in associated fungal tissues (Hynson et al., 2016), it seems likely that the high ^{15}N enrichment of these individuals was due to simultaneous association with ECM and WD fungi, but more replicates are required to evaluate the mixed C gain of *E. altissima*.

Conclusion

This study is the first to demonstrate that the largest mycoheterotrophs, *E. altissima*, is associated with a wide range of wood- and soil-inhabiting fungi, the majority of which are WD taxa. Additional associations with ECM and orchid mycorrhizal fungi imply a lack of fungal specificity in *E. altissima*, and this study provides clear evidence of a mycorrhizal generalist that targets diverse lineages of WD fungi. Although most of the WD fungi detected in this study have never been found from plant roots as mycorrhizal fungi previously, the successful symbiotic germination *in vitro* confirms their mycorrhizal ability in this orchid.

538 The measurement of C and N stable isotope natural abundances showed that *E. altissima* is a
539 full mycoheterotroph whose C originates mainly from WD fungi rather than ECM fungi.
540 Woody debris is a large store of C in forest biomass, and WD fungi play a crucial role in the
541 C cycling involved in such woody resources (Stockland et al., 2012). By associating with a
542 diverse range of WD fungi, *E. altissima* can access this large C pool, which has probably been
543 important for the evolution of such a large mycoheterotrophic plant.
544

545 **Acknowledgements**

546

547 The authors thank A. Abe, H. Enokimoto, I. Ganaha, T. Goto, K. Kaburaki, S. Katsuki, Y.
548 Kawazoe, A. Kinoshita, K. Minemoto, Y. Sakamoto, T. Saito, T. Terada, T. Tetsuka, K. Tone,
549 H. Yamaguchi, T. Yamaguchi for help with field work; K. Kobayashi, K. Ranmitsu for
550 technical support; N. Endo, T. Hattori and K. Sotome for help with fungal identification; T.
551 Shirouzu for valuable suggestion on this paper and C. Tiroch for technical assistance in
552 isotope ratio mass spectrometry. This work was supported by JSPS KAKENHI Grant Number
553 15K18597 and 17K07536, and Research Grant from Yakushima Environmental and Cultural
554 Foundation.
555

556 **Data Accessibility**

557

558 DNA sequences—GenBank Accession nos LC327023– LC327047, LC322331 – LC322337.
559

560 **Author contributions**

561

562 Y.O. designed the research. Y.O., H.X., M.K., M.M. and S.I. contributed to molecular
563 experiments. K.T., M.K., T.Y., Y.O. and Y.F. conducted field work and sample collection.
564 G.G. and J.M.S. performed isotopic analysis and analyzed the data. H.U. performed *in vitro*
565 works. Y.F. and H.X. conducted decay test. N.M. and S.Y. contributed to fungal identification.
566 Y.O., G.G., J.M.S. and T.Y. wrote the manuscript.

567

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Figure legends

Figure 1 Stem, root, and flower morphology of *Erythrorchis altissima*. Stems climbing on fallen dead wood (a) or on standing living trees (b). A thick and densely branched root clump (c) and thin and elongate roots (d). (e) Underground root clump (bar = 1 cm). (f) Flower of *E. altissima*.

Figure 2 Histology of the mycorrhizal root of *E. altissima*. (a) Cross section of the entire mycorrhizal root, bars = 1 mm. (b) Enlarged figure of cells colonized by mycorrhizal fungi, bars = 0.05 mm.

Figure 3 Seedlings and plantlet formation of *E. altissima* by symbiotic germination with fungal isolates. (a) Stages in development of seedlings. Stage 1: protocorms with 1–3-mm diameter. Stage 2: protocorms >3 mm or with root development, bar = 1 cm. (b) Plantlet after 240 days of culture with fungal isolate *Trichaptum* cf. *durum* (T-13).

Figure 4 (a) Enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$ as calculated for five individuals of *E. altissima* (flower stalk: square, flower: circle, non-mycorrhizal root: triangle, mycorrhizal root: inverted triangle), sporocarps of wood-decay fungi (cross) and ectomycorrhizal fungi (plus), decayed wood of *Distylium racemosum* (DW-Dr) and *Castanopsis sieboldii* (DW-Cs) (diamond) and stems of photosynthetic reference plants (Ref, n = 25, green square) collected from site S1. *Erythrorchis altissima*, sporocarps and decayed wood collected from *D. racemosum* and *C. sieboldii* are shown in blue with black margin and red, respectively.

Decayed wood samples were collected from host trees of each *E. altissima* individual. (b) Non-metric multidimensional scaling (NMDS) plot based on the Bray-Curtis dissimilarity matrix calculated from enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$ and N concentration data for samples collected from *D. racemosum* (blue-colored) and *C. sieboldii* (red-colored) (n = 21). Fitted vectors display the response variables $\varepsilon^{13}C$, $\varepsilon^{15}N$, and N concentration in the ordination space and indicate the differences between the groups in association with these variables. Stress = 0.02, 100 permutations; MANOVA $R^2 = 0.343$, $P = 0.001$.

Supporting information

Figure S1 Study sites of *Erythrorchis altissima* shown in Table 1.

Figure S2 Enrichment factors $\varepsilon^{13}C$ and $\varepsilon^{15}N$ calculated based on leaves of reference plants.

Table S1 Studies of *in vitro* symbiotic germination of *E. altissima.*

Table S2 List of fungal OTUs detected from *E. altissima* roots.

Table S3 List of ascomycetes fungi detected from *E. altissima* roots.

Table S4 Number of samples for isotopic analysis.

Table S5 Mean (± 1 SD) $\delta^{13}C$ and $\delta^{15}N$ values, total N and C concentrations of flowers, roots, leaves or stems of *E. altissima* and reference plants.

Table 1 Samples of *E. altissima* used for fungal identification. Location, sampling year, number of individuals and roots, and voucher number at each sampling site are listed

Site		Location	Sampling year	No. of individuals	No. of roots	Voucher
Warm-temperate area	S1	Tanegashima Is., Kagoshima, Japan	2013, 2014, 2015	9	91	TNS8505855
	S2	Tanegashima Is., Kagoshima, Japan	2005	1	5	TNS8505147
	S3	Kuchinoerabu Is., Kagoshima, Japan	2013	1	2	–
Subtropical area	S4	Kunigami, Okinawa, Japan	2007	1	1	TNS8501221
	S5	Kunigami, Okinawa, Japan	2013	2	10	–
	S6	Okinawa-city, Okinawa, Japan	2015, 2016	12	41	TNS8505854

Is. = Island

Table 2 Occurrence of fungal OTUs in each individual of *E. altissima* at warm-temperate sites

Putative taxonomic identity	Site	S1													S2	S3
	Individual ID	Y159	Ea3			Y161	Y162	D113	Ea4			Ea4D	Ea6	Ea10	Y05-10	C396
	Year collected		2013	2014	2015				2013	2014	2015					
	Host tree	Cs	Dr	Dr	Dr	Dr	none	Cs	Dr	Dr	Dr	none	Ej	Cs	Ez	Cs
	(stage)	(F)	(F)	(F)	(F)	(L)		(L)	(F)	(F)	(F)		(S)	(S)	(S)	(L)
	Decay Class	N	3	3	N	1	–	N	2~3	3	3~4	–	3	N	N	1~3
Sebacinales	Serendipitaceae sp.1														1	
Trechisporales	*Sistotremastrum* sp.1														2	
	Hyphodontia sp.1												2			
	Trichaptum cf. *durum*		11	2	2				11	2	3					
Polyporales	*Ceriporia* sp.1	2					**8**					**4**				
	Ischnoderma sp.1														1	
	Phanerochaete sp.2							2								
	Phlebia sp.1															2
	Phlebia sp.2				2								2			
	Phlebia sp.3													**3**		
	Phlebia sp.4										1					
Corticiales	*Vuilleminia* sp.1		2													
Russulales	*Russula* sp.1					**5**										
	Russula sp.2	**2**														
	Scytinostroma sp.1													**3**		
	Coniophorafomes matsuzawae							2								
Agaricales	*Gymnopus* sp.1					4			2	1						
	Hypholoma sp.1													**8**		
	Mycena sp.1							2								
Atheliales	*Athelia* sp.1														1	
Not detected						1							2			

Numbers in brackets indicate the number of root samples in which the respective fungus was detected. The root samples collected from underground are shown in bold. Host tree species (Cs = *Castanopsis sieboldii*, Dr = *Distylium racemosum*, Ej = *Elaeocarpus japonicus*, Ez = *Elaeocarpus zollingeri*) and the stage of the trees (F = Fallen dead trunk, S = Standing dead trunk, L = Living tree) are shown. The stems of Y162 and Ea4D were creeping on the ground without the host tree. The root samples of Ea3 and Ea4 were collected annually between 2013 and 2015. The level of decay of host trees was categorized into five classes as described by Fukasawa et al. (2009). N means that no data were available. The root samples from which we failed to obtain PCR products are shown as "Not detected".

Table 3 Occurrence of fungal OTUs in each individual of *E. altissima* at subtropical sites

Putative taxonomic identity	Site	S4	S5		S6											
	Individual ID	Y07-18	K58-1	K58-2	Ea12	Ea21	Ea23	Ea24	Ea28	Ea29	Ea30	Ea31	Ea35	Ea37	Ea45	Ea63
	Host tree (stage)	N	Cs (S)	Cs (S)	Cs (S)	Ms (S)	Sb (S)	Cs (L)	Cs (F)	none	Cs (L)	Cs (S)	Cd (F)	Cs (L)	N	Cs (F)
	Decay Class	N	N	N	1	N	N	N	N	–	N	N	3	N	–	N
Cantharellales	*Tulasnella* sp.1		2													
	Ceratobasidiaceae sp.1												2	2		
Trechisporales	*Trechispora* sp.1	1														
	Trechisporales sp.1		4													
	Trechisporales sp.2								1							
Hymenochaetales	*Fuscoporia* sp.1			4												
	Hymenochaetaceae sp.1					2										
Polyporales	*Phanerochaete* sp.1		2													
	Phanerochaete sp.3									2					1	
	Phanerochaetaceae sp.1															2
	Phlebia sp.2												5		3	
	Phlebia sp.5				4											
	Phlebiopsis sp.1						2									
	Stereum sp.1							2								
	Microporus sp.1											1			1	
	Hyphoderma sp.1										1					
Rssulales	*Asterostroma* sp.1									3						
Agaricales	*Neonothopanus* sp.1														1	
Not detected								1	1		1	2	1			

Numbers in brackets indicate the number of root samples in which the respective fungus was detected. Host tree species (Cs = *Castanopsis sieboldii*, Ms = *Myrsine seguinii*, Sb = *Syzygium buxifolium*, Cd = *Cinnamomum daphnoides*) and the stage of the trees (F = Fallen dead trunk, S = Standing dead trunk, L = Living tree) are shown. The stems of Ea29 were creeping on the ground without the host tree. The level of decay of host trees was categorized into five classes as described by Fukasawa et al. (2009). N means that no data were available. The root samples from which we failed to obtain PCR products are shown as "Not detected". Two root samples from K58-1 generated two fugal OTUs from each sample.

Table 4 Results of co-culture of *E. altissima* seeds with fungal isolates. Information about fungal isolates used for culture, percentage weight loss of sawdust exposed for each fungal isolate, and the number of individuals germinated by the co-culture are shown. Fungal isolate numbers, putative taxonomic identity, and NBRC numbers are listed. All isolates were extracted from *E. altissima* roots collected from site S1 in 2013

Isolate	Putative taxonomic identity	NBRC No.	Weight loss of sawdust (%)	No. of individuals Stage 1	No. of individuals Stage 2
T-13	*Trichaptum* cf. *durum*	110364	41.3 ± 2.0	2.7 ± 3.2a	0.3 ± 0.7a
T-22	*Gymnopus* sp.1	110366	18.1 ± 3.6	0	0
T-31	*Hyphodontia* sp.1	110368	43.5 ± 1.5	0	0
T-36	*Vuilleminia* sp.1	110369	24.6 ± 4.5	16.8 ± 12.3b	6.7 ± 7.9b
T-40	*Ceriporia* sp.1	110370	4.1 ± 0.5	0	0

Values are shown as means ± SD. Different letters indicate significant differences between the inoculated fungal isolates in each stage ($P < 0.05$, Wilcoxon rank-sum test). The ITS sequences of all isolates completely matched those directly amplified from root samples listed in Table 2. All isolates were deposited to the NITE Biological Resource Center (NBRC) of the National Institute of Technology and Evaluation of Japan. Seed germination was recorded two months after sowing and assigned to two germination stages: stage 1 involved rupture of the testa by the enlarged embryo and included protocorms less than 3 mm in diameter; stage 2 included non-rooted protocorms above 3 mm in diameter or rooted protocorms.

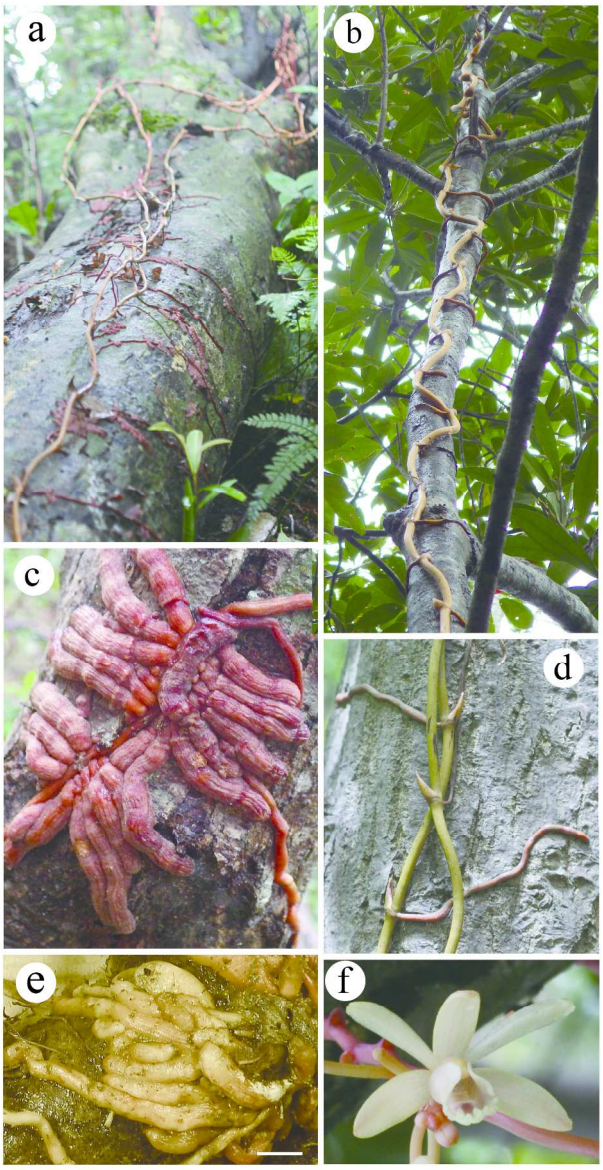


Figure 1 Stem, root, and flower morphology of Erythrorchis altissima. Stems climbing on fallen dead wood (a) or on standing living trees (b). A thick and densely branched root clump (c) and thin and elongate roots (d). (e) Underground root clump (bar = 1 cm). (f) Flower of E. altissima.

141x273mm (300 x 300 DPI)

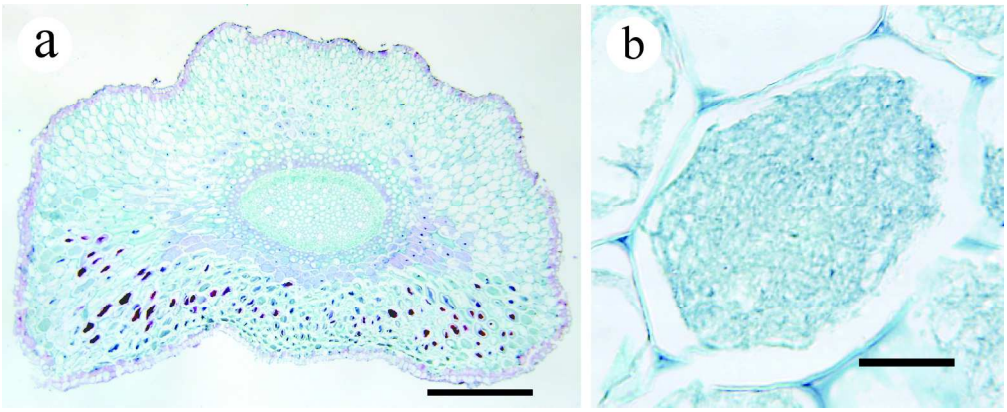
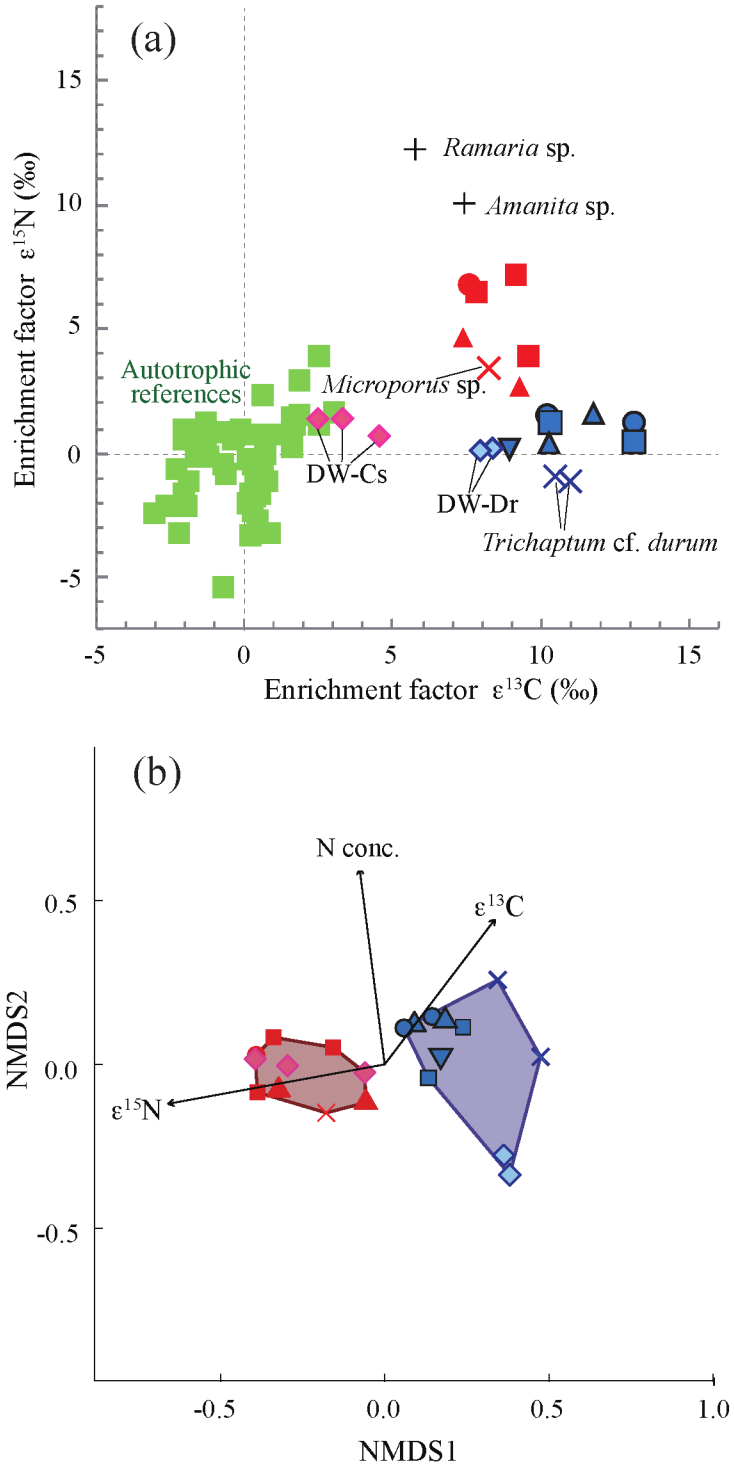


Figure 2 Histology of the mycorrhizal root of E. altissima. (a) Cross section of the entire mycorrhizal root, bars = 1 mm. (b) Enlarged figure of cells colonized by mycorrhizal fungi, bars = 0.05 mm.

205x82mm (300 x 300 DPI)

Figure 3 Seedlings and plantlet formation of E. altissima by symbiotic germination with fungal isolates. (a) Stages in development of seedlings. Stage 1: protocorms with 1–3-mm diameter. Stage 2: protocorms >3 mm or with root development, bar = 1 cm. (b) Plantlet after 240 days of culture with fungal isolate Trichaptum cf. durum (T-13).

203x75mm (300 x 300 DPI)


(a)
Ramaria sp.
Amanita sp.
Autotrophic references
Microporus sp.
DW-Cs
DW-Dr
Trichaptum cf. durum
Enrichment factor ε15N (‰)
Enrichment factor ε13C (‰)
15
10
5
0
-5
-5
0
5
10
15
(b)
N conc.
ε13C
ε15N
NMDS2
NMDS1
0.5
0.0
-0.5
-0.5
0.0
0.5
1.0