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ORIGINAL PAPER

Epipactis helleborine shows strong mycorrhizal preference towards ectomycorrhizal fungi with contrasting geographic distributions in Japan

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Abstract *Epipactis helleborine* (L.) Crantz, one of the most widespread orchid species, occurs in a broad range of habitats. This orchid is fully myco-heterotrophic in the germination stage and partially myco-heterotrophic in the adult stage, suggesting that a mycorrhizal partner is one of the key factors that determines whether *E. helleborine* successfully colonizes a specific environment. We focused on the coastal habitat of Japanese *E. helleborine* and surveyed the mycorrhizal fungi from geographically different coastal populations that grow in Japanese black pine (*Pinus thunbergii* Parl.) forests of coastal sand dunes. Mycorrhizal fungi and plant haplotypes were then compared with those from inland populations. Molecular phylogenetic analysis of large subunit rRNA sequences of fungi from its roots revealed that *E. helleborine* is mainly associated with several ectomycorrhizal taxa of the Pezizales, such as *Wilcoxina, Tuber*, and *Hydnotrya*. All individuals from coastal dunes were exclusively associated with a pezizalean fungus, *Wilcoxina*, which is ectomycorrhizal with pine trees growing on coastal dunes. *Wilcoxina* was not detected in inland forests. Coastal populations were indistinguishable from inland populations based on plant *trnL* intron haplotypes. Our results indicate that mycorrhizal association with geographically restricted pezizalean ectomycorrhizal fungi is a key control upon this orchid species' distribution across widely different forest habitats.

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Introduction

The habitats of plants range widely even within a single species, and plants use various mechanisms to colonize and survive in a specific environment (Daubenmire 1974; Larcher 2003). Since mycorrhizal fungi enable plants to access organic and inorganic sources of nutrition that are difficult for plants to gain by themselves (Smith and Read 1997; Aerts 2002), mycorrhizal associations are expected to play a crucial role in plant colonization. Although it seems certain that the mycorrhizal association is one of the key mechanisms for plants to colonize a new environment, our knowledge about the role of mycorrhizal association in plant colonization is still limited.

Epipactis helleborine (L.) Crantz, one of the most widespread orchid species in the world, has radiated extensively throughout Eurasia and North Africa (Delforge 1995) and has widely invaded North America (Luer 1975). This species occurs in a broad range of habitat types, such as dense forest floors, urban areas, open grasslands with scattered trees and calcareous soils from temperate to boreal zones (Salmia 1986; Buttler 1991; Delforge 1995; Hollingsworth and Dickson 1997). Although this species has been studied using various morphological, genetic and botanical approaches (Scacchi et al. 1987; Light and MacConaill 1991; Tyteca and Dufrene 1994; Squirrell et al. 2001; Ehlers et al. 2002; Brzosko et al. 2004), the mechanism ensuring its habitat diversity is poorly understood. All orchids, including *E. helleborine*, are complete myco-heterotrophs that depend upon mycorrhizal fungi for their carbon supply during germination and early develop-

mental stages because their small, dust-sized seeds contain minimal nutritional reserves (Arditti et al. 1990; van der Kinderen 1995; Arditti and Ghani 2000). After development of green leaves, terrestrial orchids become autotrophs that fix carbon by photosynthesis. However, the nutritional mode of *E. helleborine* is partial myco-heterotrophy; the plants depend upon both photosynthesis and mycorrhizal fungi for their carbon supply (Bidartondo et al. 2004). Such dependence on mycorrhizal fungi suggests that the mycorrhizal partner may be one of the key factors to determine whether *E. helleborine* can thrive in a specific environment.

Although *E. helleborine* typically appears in mountainous areas, it occasionally occurs in coastal dunes (Weijer 1952; Pedersen and Ehlers 2000). Japanese *E. helleborine* also appears commonly in cool mountainous areas, while the coastal populations occur in Japanese black pine (*Pinus thunbergii* Parl.) forest (Makino 1918; Maekawa 1971; Satomi 1982) that is widespread naturally and is also planted artificially along coastal sand dune (Satake 1989; Vidakovic 1991). In warm temperate areas of Japan, *E. helleborine* often appears in alpine or subalpine regions and has never been found in low-altitude forests except coastal pine. In this study, we focused on the coastal habitat of Japanese *E. helleborine* and investigated the correlation between its habitat and mycorrhizal fungi. We surveyed mycorrhizal fungi from geographically different coastal populations and compared these fungi with those from other habitats. Coastal *E. helleborine* is occasionally separated at the variety level as *E. helleborine* var. *sayekiana* and other populations inland are recognized as *E. helleborine* var. *papillosa* because of the habitat differences (Hashimoto 1987). Thus, we also compared plant chloroplast and nuclear DNA sequences between coastal and inland populations to examine whether the plant genotype is correlated with habitat. We recognize these two varieties as a complex in this study because these taxa cannot be distinguished clearly by morphological characters.

Materials and methods

Sampling

Roots of *Epipactis helleborine* were obtained from a total of twelve plants from seven seaside populations (5–400 km apart) and a total of 30 plants from 12 inland populations (10–900 km apart) between 2005 and 2007 (Table 1). Leaves or flowers were also collected for plant DNA analysis. Collected roots were sectioned with a razorblade after thorough washing in water, and fungal colonization was confirmed with a compound microscope. Mycorrhizal tissues were surface-sterilized with 0.25% NaClO for 2 min and kept at −80°C or dried in silica gel. Voucher specimens of *E. helleborine* were deposited with the Herbarium, National Museum of Nature and Science, Tsukuba, Japan (TNS765889, 765892, 765893, 765917–765919, 771101–771115, 8500019, 8500034, 8500035).

Molecular identification of mycorrhizal fungi

DNA was extracted from root tissues using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Fungal internal transcribed spacer (ITS) and large subunit (LSU) rDNA sequences were amplified using primer combinations ITS1F/ITS4 (Gardes and Bruns 1993; White et al. 1990) and LR0R/LR5 (Moncalvo et al. 2000), respectively. PCR amplification and sequencing were carried out as described by Ogura-Tsujita and Yukawa (2008). PCR

Table 1 Samples of Japanese *Epipactis helleborine* used in this study

	Sample name	Site	No. of individuals	No. of root tips
Coast	C-1	Ibaraki Pref., in *Pinus thunbergii* forest, coastal dunes	4	10
	C-2	Ibaraki Pref., in *Pinus thunbergii* forest, coastal dunes	1	2
	C-3	Chiba Pref., in *Pinus thunbergii* forest, coastal dunes	2	3
	C-4	Chiba Pref., in *Pinus thunbergii* forest, coastal dunes	3	4
	C-5	Kanagawa Pref., in *Pinus thunbergii* forest, coastal dunes	5	18
	C-6	Akita Pref., in *Pinus thunbergii* forest, coastal dunes	3	8
Inland	I-1	Hokkaido Pref., in mixed conifer forest	3	4
	I-2	Hokkaido Pref., in mixed conifer forest	1	4
	I-3	Hokkaido Pref., in *Abies sachalinensis* forest	2	8
	I-4	Hokkaido Pref., in *Abies sachalinensis* forest	2	6
	I-5	Iwate Pref., in *Quercus crispula* forest	2	8
	I-6	Iwate Pref., in calcareous soil, near oakwood	1	4
	I-7	Iwate Pref., in mixed birch forest	4	14
	I-8	Nagano Pref., in calcareous soil, near deciduous forest mixed with pine	1	2
	I-9	Nagano Pref., in grassland, scattered *Larix kaempferi*	2	8

Pref. Prefecture

products that were difficult to sequence directly were cloned using the pGEM-T Vector System II (Promega, Madison, WI, USA). GenBank (http://www.ncbi.nlm.nih.gov) accession numbers of the sequences of mycorrhizal fungi determined in this study are shown in Fig. 1 and Supplementary Fig. 1. Sequences were analyzed using a BLAST search (Altschul et al. 1997) against the NCBI sequence database (National Center for Biotechnology Information, GenBank) to find the closest sequence matches in the GenBank database.

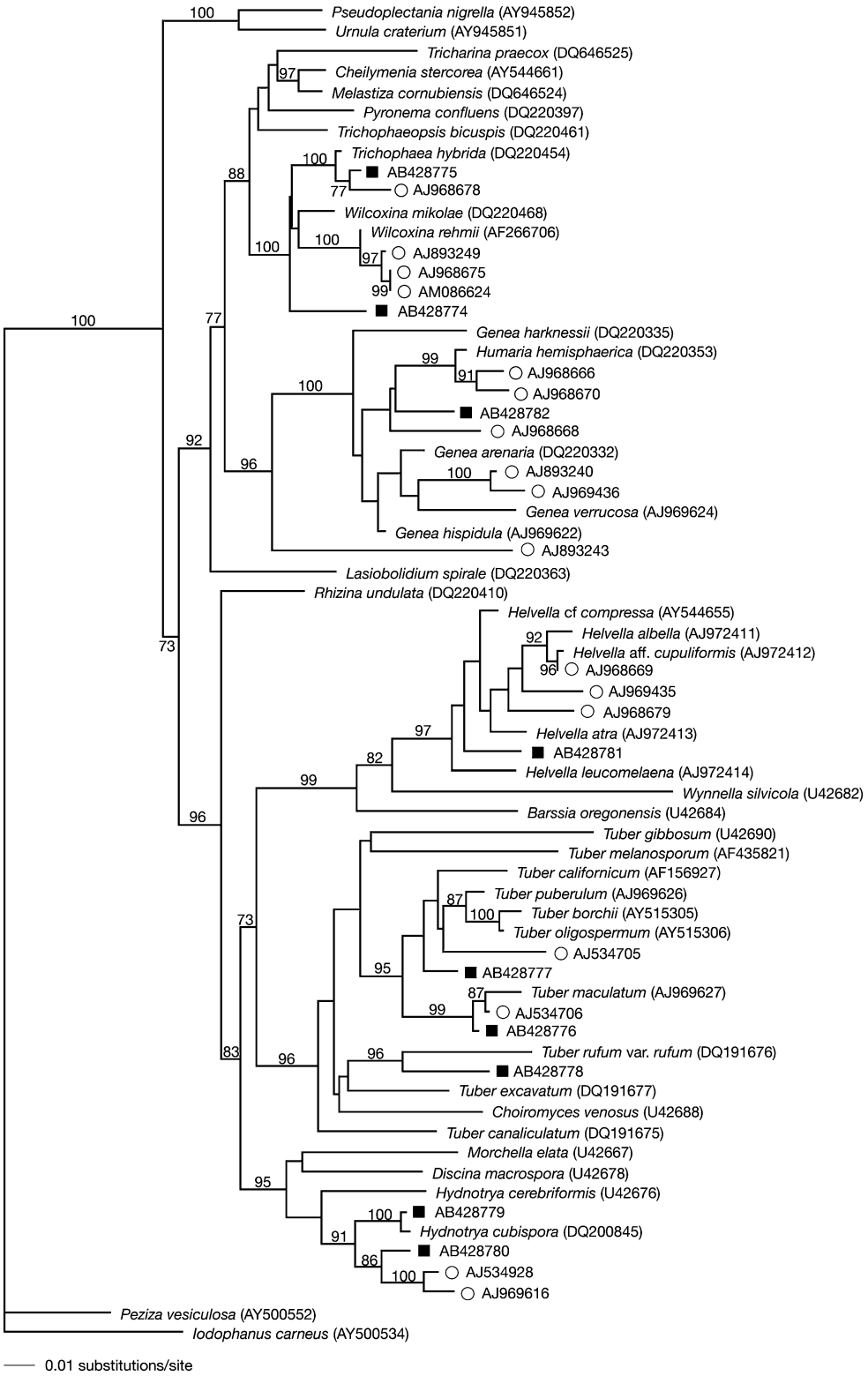

Fig. 1 Phylogenetic placement of mycorrhizal fungi of *Epipactis helleborine* (*black squares*) based on large subunit rDNA sequences of Pezizales available in GenBank. Sequences of ectomycorrhizal fungi (ECM) from GenBank are shown as open circles and all GenBank accessions are shown to the right. Phylogenetic analysis was conducted using neighbor-joining with 1,000 bootstrap replicates (values greater than 70% are shown)

Plant DNA analysis

DNA was extracted from fresh leaves or flowers using a DNeasy Plant Mini Kit. The *trnL* intron, *trnK* intron, *matK* and *rpL16* introns of chloroplast DNA and the ITS region of nuclear DNA were respectively amplified as described by Hidayat et al. (2005) using the following primer combinations: primers c/f (Taberlet et al. 1991), trnK-3914F/OMAT1R (Johnson and Soltis 1995; Hidayat et al. 2005), OMAT1F/trnK-2R (Johnson and Soltis 1995; Yukawa et al. 1999), F71/R1661 (Jordan et al. 1996) and 17SE /26SE (Sun et al. 1994). The purification and sequencing of the PCR products followed the method described by Ogura-Tsujita and Yukawa (2008). Our preliminary comparison of the sequences between individuals from coastal (C-1) and inland (I-8) populations found that the individuals from C-1 had three substitution sites and one deletion site in the *trnL* intron, one substitution site in *matK* and no polymorphisms in the *rpL16* intron or ITS sequences. Hence, the *trnL* intron sequences were used for further analysis. The GenBank accession numbers of the plant sequences reported in this paper are AB428732–AB428736.

Phylogenetic analysis

The LSU sequences of each ITS type (Table 2) and the ITS sequences of *Wilcoxina* types 1 and 2 were used for analysis. Sequences were aligned using Clustal X software (Thompson et al. 1997), followed by manual adjustment. Phylogenetic analyses were conducted with PAUP* version 4.0b6 software (Swofford 2001). Distance trees were obtained using the neighbor-joining (NJ) method (Saitou and Nei 1987) with a Kimura two-parameter correction (Kimura 1980). For assessing the relative robustness of branches, the bootstrap method (Felsenstein 1985) was used with 1,000 replicates. LSU sequences were initially analyzed with the data matrix employed in Hansen and Pfister (2006), which clarified the phylogenetic relationship of the Pezizales, and these sequences belonged to the clade of Pyronemataceae, Tuberaceae, Discinaceae, and Helvellaceae. The sequences of related species found from Tedersoo et al. (2006) and Perry et al. (2007) were added to these results for the analysis. The LSU sequences of *Peziza vesiculosa* and *Idophanus carneus* were selected as the outgroup taxa.

To determine the detailed phylogenetic placement of *Wilcoxina* types from the mycorrhizal roots of the coastal populations, ITS sequences of *Wilcoxina* types 1 and 2 were used. These ITS sequences were initially combined with LSU sequences and analyzed with the sequences (encompassing 18S, ITS1, and 28S) employed in Egger (1996) that clarified the phylogenetic relationship of *Wilcoxina* species. These results showed that *Wilcoxina* types from *E. helleborine* and *Wilcoxina mikolae* became a sister group to *Wilcoxina rehmii*. From these results, ITS sequences of *Wilcoxina* types (encompassing the ITS1, 5.8S, and ITS2 regions) were analyzed with the closest

Table 2 Patterns of fungal ITS sequence types from mycorrhizal roots and plant haplotypes in *Epipactis helleborine*

Sample Name	Fungal type												Plant Haplotype
	Wilcoxina		*Tuber*			*Hydnotrya*		*Helvella*	*Genea*	*Exophiala*	Others	Undetected	
	1	2	1	2	3	1	2						
Coast													
C-1	8[a]	–	–	–	–	–	–	–	–	–	2	–	I
C-2	2	–	–	–	–	–	–	–	–	–	–	–	I
C-3	2	–	–	–	–	–	–	–	–	–	–	1	I
C-4	4	–	–	–	–	–	–	–	–	–	–	–	I
C-5	17	–	–	–	–	–	–	–	–	–	1	–	I
C-6	3	4	–	–	–	–	–	–	–	–	1	–	II
Inland													
I-1	–	–	–	–	–	1	–	–	–	2	–	1	I, V
I-2	–	–	1	–	–	–	–	–	–	–	–	3	IV
I-3	–	–	7	–	–	–	–	–	–	1	–	–	IV
I-4	–	–	3	–	–	–	–	–	–	2	1	–	IV
I-5	–	–	–	3	–	–	–	–	–	–	5	–	II
I-6	–	–	–	–	3	–	–	–	–	–	–	1	III
I-7	–	–	–	–	–	–	8	4	–	–	2	–	I, II
I-8	–	–	–	–	–	–	–	–	2	–	–	–	III
I-9	–	–	–	–	–	8	–	–	–	–	0	–	II

[a] Number of root tips for each ITS type detected. The dominant ITS type was preferentially counted when more than two fungal clones were detected from one root tip.

matching sequences in the GenBank database. The sequences of *W. rehmii* were used as the outgroup taxon because no sequences were found for an appropriate outgroup to the genus *Wilcoxina*.

Results

A total of 103 orchid roots were analyzed and fungal ITS sequences were successfully obtained from 97 root tips. The predominant fungal sequences were classified into ten ITS types, i.e., *Wilcoxina* (2), *Tuber* (3), *Hydnotrya* (2), *Helvella* (1), *Genea* (1) and *Exophiala* (1), by BLAST analysis (Table 2). All ITS types were the dominant fungi in the roots of *E. helleborine*, except for *Exophiala*, which co-occurred with other ITS types. Mycorrhizal fungi of the coastal populations were restricted to *Wilcoxina* and other fungal types were found from the inland individuals. Besides these ITS types, *Leptodontidium*, *Ceratobasidium*, *Cenoccocum*, *Russula*, *Nectria*, and *Trichoderma* were also found. Root samples in which fungal sequences were not detected produced only plant DNA sequences.

Since all ITS types except *Exophiala* belonged to the Pezizales, the LSU sequences from each ITS type were analyzed with representative sequences belonging to the Pezizales from GenBank to confirm their phylogenetic placement (Fig. 1). All LSU sequences are nested within the Pyronemataceae, Tuberaceae, Helvellaceae, and Discinaceae and are closely related to the sequences of pezizalean ectomycorrhizal fungi in GenBank. *Wilcoxina* 1 and 2 form a monophyletic group with *W. mikolae* and *W. rehmii* with 100% bootstrap support (BS). Additionally, the ITS sequence of *Wilcoxina* 2 is 99% identical to that of an ectomycorrhizal fungus of *P. thunbergii* (AB250949). *Tuber* 1 and 2 are nested among *Tuber* species (95% BS) and *Tuber* 3 clusters with *Tuber rufum* var. *rufum* (96% BS). The ITS region of *Tuber* 1 showed near identity (99%) to the ectomycorrhizal fungus of *Tilia cordata* (AJ534706). *Hydnotrya* 1 forms a distinct lineage with *Hydnotrya cubispora* (100% BS), sharing 99% LSU sequence identity. *Hydnotrya* 2 and two fungal sequences from ectomycorrhizae in GenBank form a sister group to *Hydnotrya* 1. The ITS sequences of *Hydnotrya* 1 and 2 are, respectively, 84 and 89% identical to an ectomycorrhizal fungus of *Fagus sylvatica* (AJ969616). The ITS sequence of *Hydnotrya* 2 is 93% identical with that of an ectomycorrhizal fungus of *Abies homolepis* (AB218071). The sequence of the *Helvella* type is nested within the *Helvella* lineage (97% BS) and its ITS sequence is 85% identical with that of a *Helvella elastica* ascocarp (AF335455). The *Genea* type is nested within lineages of *Genea* and *Humaria* species and the ITS sequence of the *Genea* type closely matched that of a pezizalean ectomycorrhizal fungus (AJ968670; 83% identical) by BLAST analysis.

The ITS sequences of *Wilcoxina* 1 and 2 were analyzed with closely related sequences from GenBank to resolve deep-level phylogenetic relationships (Supplementary Fig. 1). *Wilcoxina* 2 forms a monophyletic group with an ectomycorrhizal fungus of *P. thunbergii* (AB250949; 100% BS) and is closely related to the ectomycorrhizal fungus of *Picea abies* (DQ069002). *Wilcoxina* 1 is highly supported as a sister taxon to the clade, which includes *Wilcoxina* 2 (93% BS). *Wilcoxina* 1 and 2 form a sister group with *W. mikolae* (100% BS) from ectomycorrhizal tissues of *Pinus* and *Picea* species.

The plant haplotypes were classified into five distinct types using plastid *trnL* intron sequences; one or two haplotypes were observed in each population (Table 2). Coastal populations consisted of haplotypes I and II while inland populations contained all haplotypes detected. Coastal populations were indistinguishable from inland populations based on plant haplotypes.

Discussion

The results of the present study show that *E. helleborine* is mainly associated with several taxa of the pezizalean ectomycorrhizal fungi. Phylogenetic analysis of fungal LSU sequences from *E. helleborine* revealed that all dominant fungal types were included within the Pezizales, especially the Pyronemataceae, Tuberaceae, Helvellaceae, and Discinaceae (Fig. 1). Additionally, the LSU sequences from *E. helleborine* were closely related to those of pezizalean ectomycorrhizal fungi reported by Tedersoo et al. (2006). Within fungal ITS types, *Wilcoxina* 1 and 2 form a strongly supported monophyletic group with other *Wilcoxina* species, which are known as ectomycorrhizal fungi of Pinaceae (Yang and Korf 1985; Piché et al. 1986; Scales and Peterson 1991a,b; Baar et al. 1999; Bidartondo et al. 2001; Kernaghan et al. 2003; Fujimura et al. 2005; Menkis et al. 2005; Trocha et al. 2006). *Tuber* species, which cluster with *Tuber* 1, 2 and 3, form ectomycorrhizae with a variety of trees, such as *Quercus*, *Corylus*, *Tilia*, *Salix*, and *Picea* (Singer and Harris 1987; Sisti et al. 1998; Selosse et al. 2004; Trocha et al. 2006; Smith et al. 2007; Morris et al. 2008; Hrynkiewicz et al. 2008). Each LSU sequence of *Hydnotrya*, *Helvella*, and *Genea* from *E. helleborine* roots clusters with those of ectomycorrhizal fungi from the GenBank database. The ITS region also supports the evidence that these lineages are ectomycorrhizal fungi since their closest sequence matches in the GenBank database always include ITS sequences from ectomycorrhizal roots. *Exophiala* was detected at three sites but was not a dominant fungus within any individual or site

(Table 2). The ITS sequences of *Exophiala* closely matched those of an endophytic fungus (EF495231, 99% identity) and a fungal isolate from orchid roots (AY833042, 99% identity), suggesting that *Exophiala*, and also other minor fungi from *E. helleborine*, were probably present as minor endophytes, spores, or rhizosphere hyphae. Failure to obtain fungal PCR products from a small number of roots may be due to poor colonization or primer mismatch.

Coastal individuals were specifically associated with *Wilcoxina*. A total of 18 plants from six coastal sites were analyzed and *Wilcoxina* 1 or 2 were identified from all individuals (Table 2). Phylogenetic analysis of *Wilcoxina* ITS sequences showed that the fungal sequences from *E. helleborine* are highly similar to those from ectomycorrhizal fungi of *Pinus* and *Picea* species (Supplementary Fig. 1). In particular, the ITS sequence of *Wilcoxina* 2 is 99% identical to that of a mycorrhizal fungus from *P. thunbergii*. These results suggest that *Wilcoxina* 1 and 2 are primarily distributed along the Japanese coast by forming an ectomycorrhizal association with *P. thunbergii*, and *E. helleborine* grows only through association with these fungi.

In our data, the coastal populations do not differ genetically from the inland populations at the *trnL* intron sequence level. The distribution of the *trnL* intron haplotypes showed that coastal individuals consisted of two haplotypes but these haplotypes were also found in inland populations (Table 2). Plant haplotypes I and II from coastal populations were exclusively associated with *Wilcoxina*, while these haplotypes from inland populations were associated only with *Tuber*, *Hydnotrya*, or *Helvella*.

Our results demonstrate that mature Japanese *E. helleborine* have a strong preference for several taxa of pezizalean ectomycorrhizal fungi. Similarly, it was shown that *Tuber* and *Wilcoxina* are the dominant fungi in roots of European and American *E. helleborine* (Bidartondo et al. 2004). These results suggest that mycorrhizal association with pezizalean ectomycorrhizal fungi is common among *E. helleborine* worldwide. Secondly, we found that *E. helleborine* growing on coastal dunes was exclusively associated with the *Wilcoxina* species group, suggesting that *E. helleborine* successfully colonizes Japanese coastal dunes by associating with *Wilcoxina*. This hypothesis can be tested via in situ germination experiments. In our data, plant *trn*L intron haplotypes did not discriminate between coastal and inland individuals. All of our results indicate that the plant characteristics concerned with the mycorrhizal association determine the habitat pattern of *E. helleborine*. The results of Bidartondo et al. (2004) also suggest that *E. helleborine* associates with the ectomycorrhizal fungi of forest trees and successfully thrives in the low-irradiance understory of forests with nutritional supplementation from its mycorrhizal partners. Mycorrhizal association with pezizalean ectomycorrhizal fungi appears to be the mechanism behind how the globally widespread orchid, *E. helleborine*, colonizes its widely ranging habitat.

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