

# *Pyrola japonica*, a partially mycoheterotrophic Ericaceae, has mycorrhizal preference for russulacean fungi in central Japan

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Received: 1 April 2016 / Accepted: 14 June 2016 / Published online: 21 June 2016  
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**Abstract** Mycorrhizal symbiosis often displays low specificity, except for mycoheterotrophic plants that obtain carbon from their mycorrhizal fungi and often have higher specificity to certain fungal taxa. Partially mycoheterotrophic (or mixotrophic, MX) plant species tend to have a larger diversity of fungal partners, e.g., in the genus *Pyrola* (Monotropoideae, Ericaceae). Preliminary evidence however showed that the Japanese *Pyrola japonica* has preference for russulacean fungi based on direct sequencing of the fungal internal transcribed spacer (ITS) region from a single site. The present study challenges this conclusion using (1) sampling of *P. japonica* in different Japanese regions and forest types and (2) fungal identification by ITS cloning. Plants were sampled from eight sites in three regions, in one of which the fungal community on tree ectomycorrhizal (ECM) tips surrounding *P. japonica* was also analyzed. In all, 1512 clone sequences were obtained

successfully from 35 *P. japonica* plants and 137 sequences from ECM communities. These sequences were collectively divided into 74 molecular operational taxonomic units (MOTUs) (51 and 33 MOTUs, respectively). MOTUs from *P. japonica* involved 36 ECM taxa (96 % of all clones), and 17 of these were *Russula* spp. (76.2 % of all clones), which colonized 33 of the 35 sampled plants. The MOTU composition significantly differed between *P. japonica* and ECM tips, although shared species represented 26.3 % of the ECM tips community in abundance. This suggests that *P. japonica* has a preference for russulacean fungi.

**Keywords** Arbutoid mycorrhiza · Cloning · ITS barcoding · Mycorrhizal specificity · Pyroloid mycorrhiza · *Russula*

## Introduction

Mycorrhizal symbiosis is a mutualistic relationship in which carbohydrates from plants are exchanged against mineral nutrients from mycorrhizal fungi (Smith and Read 2008; van der Heijden et al. 2015). However, mycoheterotrophic (MH) plants, which are mostly achlorophyllous, represent exceptions to this general rule. Since they lost photosynthetic ability, both carbohydrates and minerals are obtained from associated mycorrhizal fungi (Merckx 2013). More recently, some green plants were suggested to obtain a significant proportion of fungal carbon (Selosse and Roy 2009; Selosse et al. 2016): the so-called mixotrophic (MX) plants are partially mycoheterotrophic but retain photosynthetic abilities (e.g., Gebauer and Meyer 2003; Gonneau et al. 2014).

In temperate regions, MH and MX plants evolved multiple times in the family Orchidaceae and in Monotropoideae in the family Ericaceae (Merckx et al. 2013). Molecular analyses revealed that mycorrhizal associations of MH and MX plants

**Electronic supplementary material** The online version of this article (doi:10.1007/s00572-016-0715-2) contains supplementary material, which is available to authorized users.

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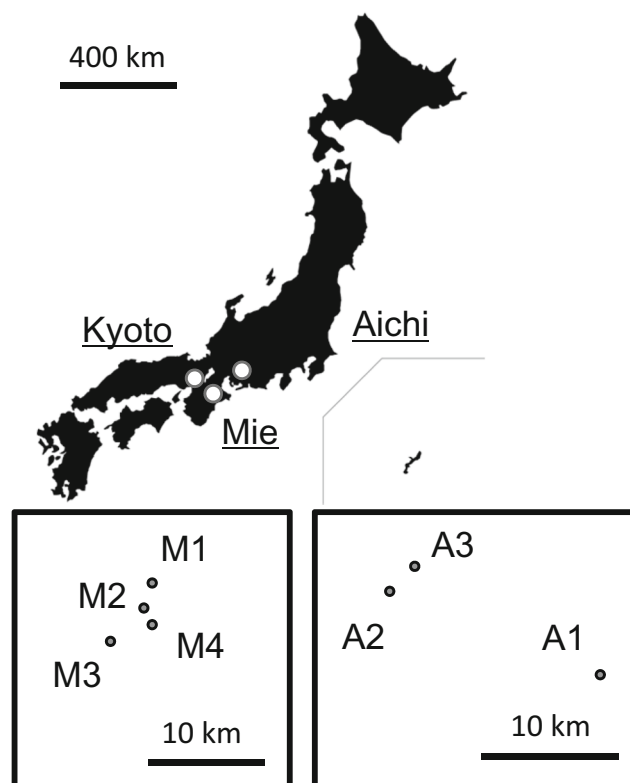
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are often biased toward certain groups of ectomycorrhizal (ECM) fungi (Matsuda et al. 2011; Merckx et al. 2013) that receive carbon from surrounding autotrophic trees they simultaneously colonize (Björkman 1960). Thus, tripartite interactions within ECM networks facilitate the coexistence of trees and MH plants (Matsuda and Hijii 2004; Selosse et al. 2006; Simard et al. 2012; Nara 2015). Most MH and many MX species associate with a narrow range of fungi (e.g., Waterman et al. 2013). Such specificity becomes even higher at a local population scale, and populations inhabiting environmentally or geographically different sites may associate with different subsets of fungi (Dowie et al. 2012; Hynson et al. 2015).

*Pyrola* (Monotropoideae, Ericaceae), a plant genus of the northern hemisphere, comprises ca. 35 species (Liu et al. 2010) with a fully MH germination stage (Hashimoto et al. 2012; Hynson et al. 2013). It contains one MH and many MX species without mycorrhizal fungal specificity (Zimmer et al. 2007; Tedersoo et al. 2007; Hynson et al. 2009). These plants form arbutoid or pyroloid mycorrhizas that are characterized by the presence of intracellular hyphal coils, while fungal mantle formation ranges from apparent to absent (Robertson and Robertson 1985; Peterson et al. 2004; Massicotte et al. 2008; Vincenot et al. 2008). A root can be infected by multiple fungal species, especially when the mantle is absent (Tedersoo et al. 2007; Toftegaard et al. 2010). Molecular identifications of pyroloid mycorrhizal fungi have revealed associations with ECM fungi and a taxonomic range including several families such as Atheliaceae, Russulaceae, and Thelephoraceae per *Pyrola* species, which are therefore generalist at adulthood (Zimmer et al. 2007; Tedersoo et al. 2007; Vincenot et al. 2008; Hynson and Bruns 2009; Toftegaard et al. 2010; Hashimoto et al. 2012).

However, unlike the other *Pyrola* species, Asian *Pyrola japonica* was suggested to have a certain mycorrhizal preference. Although multiple ECM fungal taxa associate with *P. japonica* as with other *Pyrola* species, members of *Russula* account for 84 % of the analyzed sequences (Matsuda et al. 2012). This is currently the sole example of mycorrhizal preference in *Pyrola*, and it may have evolved secondarily from ancestors with generalist relationships, as suggested for some MX Orchidaceae (e.g., Girlanda et al. 2006). However, Matsuda et al. (2012) used direct sequencing of PCR products, and the rather low rate of successful sequencing might have caused a methodological error, neglecting admixture of co-infecting fungi.

The aim of this study was to identify more precisely the diversity of the mycorrhizal community associated with 39 individuals of *P. japonica* in three regions from central Japan (Fig. 1). A cloning method for PCR products refined fungal assemblages and occurrence frequencies in roots of the plant. We hypothesized that *Russula* is the most abundant taxon associated with *P. japonica*, irrespective of habitats.



**Fig. 1** Location of sampling regions. *Insets* indicate study sites in each region. Aichi and Mie involved several sites at distances ranging from 1.8 to 15.9 km. M1 was the study site used in Matsuda et al. (2012)

Moreover, we investigated whether the communities associated with *P. japonica* simply reflected those from surrounding ECM trees or a true preference for russulacean species. We hypothesized that *Russula* is not necessarily the most abundant taxon on surrounding ECM trees but that its abundance on *P. japonica* roots reflects a preferential association.

## Materials and methods

### Study sites and sampling

Mature individuals of *P. japonica* were collected from eight sites in central Japan in 2012 and 2013 from three regions, namely sites A1–A3 in Aichi, M1–M4, in Mie, and K1 in Kyoto (Fig. 1). All sites harbor temperate deciduous or evergreen ECM forests that, despite roughly similar climatic conditions, differ by dominant ECM tree species (Table 1). No major understorey plants other than *P. japonica* were observed except the Poaceae *Pleioblastus chino* var. *viridis* at site M1 (Matsuda et al. 2012; Fig. S1). At each site, individuals were at least 20 m apart to secure independent sampling. At the site M4, we collected three plants with dried shoots because no intact healthy shoot was available at the sampling date. Plants were dug up with a 15 × 15 × 15-cm soil core to obtain the

**Table 1** Study sites

Sampling regions and sites	Site codes	Sampling date	Overstory ECM trees	No. of plants collected	Mean temperature (°C)	Annual precipitation (mm)	Distance from meteorological station (km) <sup>b</sup>
Aichi Toyota	A1	06.2013	<i>Quercus glauca</i> , <i>Pinus densiflora</i>	6 [4] <sup>a</sup>	15.2	1341	2.9
Aichi Owariasahi	A2	06.2013	<i>Castanopsis cuspidata</i>	3 [1]	16.1	1515	10.6
Aichi Seto	A3	06.2013	<i>P. densiflora</i>	3 [1]	16.1	1515	12.4
Mie Tsu no. 1	M1	06.2012	<i>Q. serrata</i> , <i>Q. acutissima</i>	6	14.6	1545	23.6
Mie Tsu no. 2	M2	06.2012	<i>Q. serrata</i>	6	14.6	1545	21.6
Mie Tsu no. 3	M3	06.2012	<i>C. cuspidata</i>	9	14.6	1545	21.8
Mie Tsu no. 4	M4	06.2013	<i>C. cuspidata</i> , <i>Q. salicina</i>	3	14.6	1545	24.5
Kyoto Nagaokakyo	K1	06.2013	<i>C. cuspidata</i>	3	16.0	1506	11.5

<sup>a</sup> Brackets indicate number of soil cores collected to investigate surrounding ECM community on tree roots

<sup>b</sup> Data on temperature and precipitation were recorded at the nearest meteorological stations in the year of sampling

whole root system and kept at 5 °C until mycorrhizal observation. We collected 18 plants in 2012 and 21 plants in 2013 (Table 1). In the laboratory, excavated soil was washed out under running tap water on a 2-mm mesh sieve. Mycorrhizal roots were excised under a stereomicroscope (up to  $\times 115$  magnification; SZX16, Olympus, Tokyo). Since *P. japonica* mycorrhizas have no fungal mantle, we defined mycorrhizas as whitish to brownish zones characterizing the occurrence of intracellular hyphal coils (Matsuda et al. 2012; Fig. S1). Up to six 2–5-mm-long mycorrhizal root fragments were recovered per core, i.e., a total of 28 and 102 root fragments (accounting for a total 140 and 204 mm of root) in 2012 and 2013, respectively. They were stored at  $-80$  °C until molecular analysis. In order to characterize ECM diversity on tree roots surrounding *P. japonica*, the surrounding ECM roots were also sampled around six plants from the Aichi sites (numbers in brackets in Table 1). From each soil core containing *P. japonica* root systems, 20 to 30 ECM tips were arbitrarily sampled and stored at  $-80$  °C until molecular analysis.

### Molecular analysis

Since the different epidermal cells of a single root fragment can associate with different mycorrhizal fungi, we cloned PCR products for barcoding. Genomic DNA was extracted from root samples using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The fungal internal transcribed spacer (ITS) region of nuclear ribosomal DNA was amplified with the *Ex Taq* Kit (TaKaRa, Ohtsu, Japan) by PCR using TaKaRa PCR Thermal Cycler Dice (Model TP600, TaKaRa, Ohtsu, Japan). For each plant sample, primer pairs ITS1f and ITS4 or ITS1f and TW13 were used for amplification, as in Matsuda et al. (2012). Positive PCR products were cloned

using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Twenty and 64 successfully inserted white colonies were picked up in 2012 and 2013, respectively. Selected colonies were further amplified with the *Ex Taq* Kit or EmeraldAmp MAX PCR Mix (TaKaRa, Ohtsu, Japan) according to the manufacturer's instructions, using the previous primer sets. Thermal cycle conditions for PCR were as follows: preliminary denaturation at 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 70 s, and final extension at 72 °C for 10 min. Positive clone products were submitted to restriction fragment length polymorphism (RFLP) analysis with *Alu* I and *Hinf* I (TaKaRa, Ohtsu, Japan). The digested fragments were separated on 4 % 3:1 Nusieve agarose gel in TAE buffer with a 100-bp DNA ladder (Bio Regenerations, Yokohama, Japan), and the length of fragments was estimated using a CS Analyzer 2.0 (Atto, Tokyo, Japan). Clones were grouped into RFLP types (but identical RFLP patterns detected from different plants were tentatively treated as different RFLP types in the first instance). One to four representative clones of each RFLP type were then sequenced. They were cleaned using Illustra GFX PCR DNA and the Gel Band Purification Kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and sequenced using ABI 3730 (Applied Biosystems, Foster City, CA, USA) after reactions with the BigDye Terminator v3.1 Cycle Sequencing Kit (Invitrogen, Carlsbad, CA, USA) with primer ITS1f. When sequencing was not successful, the samples were sequenced bidirectionally using reverse primer ITS4 or TW13.

For the analysis of ECM tips, genomic DNA was extracted by homogenizing using a pestle and mortar and heated in buffer A (Thomson and Henry 1995) at 95 °C. KOD FX (TOYOBIO, Tokyo, Japan) was used for PCR

amplification with the following thermal cycle conditions: 94 °C for 2 min, 35 cycles of 98 °C for 10 s, 55 °C for 30 s, 68 °C for 70 s, and 72 °C for 10 min. Primers were ITSOF (Tedersoo et al. 2008) and TW13 in the first trial. When the amplification was not successful, the reverse primer was changed into LB-W (Tedersoo et al. 2007). Positive PCR products confirmed through electrophoresis were purified by Illustra GFX PCR DNA and the Gel Band Purification Kit or ExoSAP-IT (Affymetrix, Tokyo, Japan) and sequenced as above.

### Data analysis

Obtained sequences were adjusted manually using the MEGA version 5.22 software (Tamura et al. 2011) and grouped into molecular operational taxonomic units (MOTUs) at a 97 % similarity cutoff using MOTHUR version 1.34.4 software (Schloss et al. 2009). The MOTU classification was validated by constructing a *p* distance tree. The representative sequences were selected by the abundance method for BLAST searches against international nucleotide sequence databases (INSD) for taxonomic placement (Altschul et al. 1997), and  $\geq 97$  % identity with known species were named as species. Putative chimera clones inferred from BLAST results were discarded manually from further analyses. Representative fungal ITS sequences were deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers LC096270 to LC096953. In the following analyses, each MOTU and the occurrence frequency of MOTU were treated as taxon and abundance, respectively. For *P. japonica* MOTUs, the accumulation curves of taxa in the Aichi and Mie regions and all sites altogether were constructed using the EstimateS version 9.1 software (Colwell 2013). In addition, Shannon's, Fisher's alpha, and Simpson's diversity indices of root-associated fungi were calculated to characterize the pattern of fungal assemblages of *P. japonica*. Non-metric multidimensional scaling (NMDS) and PERMANOVA were also performed to test if the community was structured in accordance with geographic regions. Clone abundance data of each site were used for ordination with the Horn dissimilarity index. We also used the function "envfit" implemented in the Vegan package (Oksanen et al. 2013) to identify variables that were significantly correlated with the community composition of ECM fungi. Eight variables relating to host trees, geographical location, climate, and soil conditions fitted as vectors with 999 permutations. The location was represented by the principal coordinates of neighborhood matrix (PCNM) calculated based on the distance matrix among study sites expressed as longitudinal and

latitudinal coordinates (Dray et al. 2006). For comparison with fungal assemblages, MOTU compositions of *P. japonica* and ECM tips of pooled cores collected at Aichi were tested by means of the  $\chi^2$  test. In addition, the occurrence frequency of top five MOTUs on both *P. japonica* and ECM tips was examined using Fisher's exact test. These analyses were accomplished by R 3.12 (R Core Team 2013) and the package vegan (Oksanen et al. 2013). Significance level was defined at  $p < 0.05$  unless otherwise stated. Since Russulaceae were the dominant fungal family (see "Results"), phylogenetic clustering of recovered russulacean MOTUs was estimated by reconstructing a phylogenetic tree. Representative sequences of this study along with several russulacean sequences represented in the International Nucleotide Sequence Database Collaboration (INSDc) were aligned by MAFFT 7 with the L-ins-I strategy and 1 PAM scoring matrix or otherwise with default settings (Katoh and Standley 2013). A neighbor-joining (NJ) tree of the ITS region was constructed, using *Lactarius* sequences as outgroups, based on the Maximum Composite Likelihood model with 1000 bootstrap replicates using MEGA 5.22.

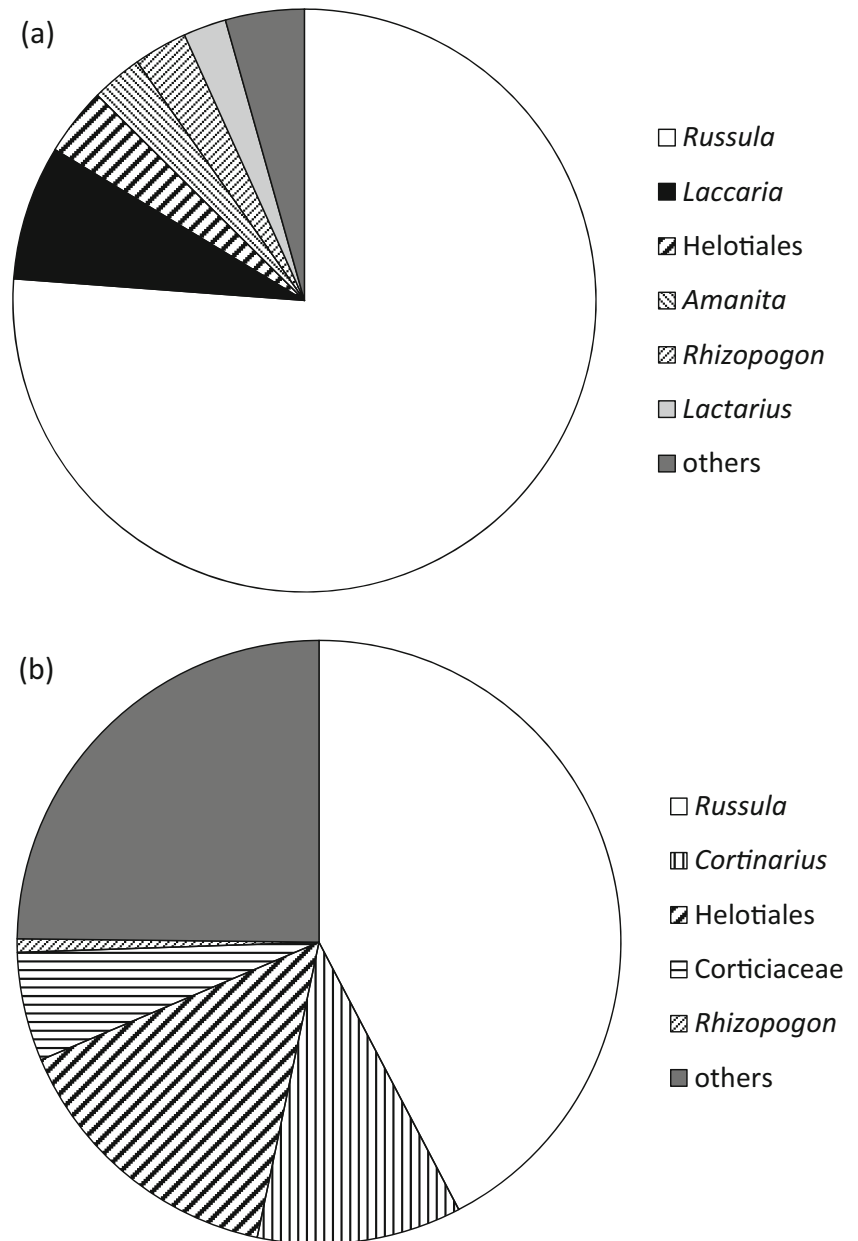
## Results

### Mycorrhizal fungi associated with *P. japonica*

PCR amplifications were successful for all *P. japonica* individual plants but one from A1, one from M3, and two from M4 (probably due to low colonization). In all, 1512 sequences from *P. japonica* were obtained out of 1706 clones, i.e., from 8 to 64 clones per individual plant. Based on a 97 % cutoff, these sequences clustered into 51 MOTUs that included 36 ECM taxa accounting for 96.0 % of all clones (Tables S1 and S2 and Fig. 2a; we included Helotiales whose status, ECM versus endophytic, is debated). Other taxa were minor in frequency (4.0 %) and matched with putatively saprotrophic or endophytic species. The accumulation curves for ECM taxa MOTUs did not saturate, when all the collected samples were pooled or when only samples in Aichi or Mie were examined (Fig. S2a). However, they saturated at the individual plant level (Fig. S2b), suggesting that new MOTUs in populations were due to differences between individuals rather than to insufficient sampling effort within individuals.

In all, 17 MOTUs belonged to the genus *Russula*, accounting for 76.2 % of all clones (Table S2 and Fig. 2a). MOTU 12 matched with *Russula densifolia* (AB291764) and MOTUs 15 and 30 with *Russula*

**Fig. 2** Percentages of occurrence rates of fungal taxa detected from **a** *Pyrola japonica*, from 35 plants successfully analyzed (1512 clones) over 3 regions, and **b** ECM tips from Aichi (137 sequences). More details are given in Tables 2 and S2 and S3



*violeipes* (KF361779 and KF361796, respectively) with more than 97 % identity, whereas other *Russula* MOTUs did not match with any described taxon at the threshold (Table S1). The 11 most abundant MOTUs belonged to *Russula*, except for the fourth, which matched with *Laccaria amethystina* (KF692083; 99 %) and accounted for 113 (7.5 %) of all clones. *Russula* MOTUs were detected from 33 plants (out of 35 for which barcoding succeeded) and dominated in 28 of these. Although half of the MOTUs were shared among plants within sites or a region, only two MOTUs (*Russula* spp. 2 and 7) were found in different regions.

*L. amethystina* occurred in 12 plants from 5 sites and 2 regions and dominated in 3 plants where it accounted for 47 to 100 % of clones. Finally, three individual plants were respectively dominated by *Amanita ceciliae*, *Rhizopogon* sp. 1, and Helotiales sp. 1 that accounted for 72, 69, and 43 % of their respective clones. Other MOTUs, especially saprotrophic or endophytic, were less abundant in all or individual plants (<2 % of all clones) and were mostly detected from a single site or individual (Table S2). Shannon's, Fisher's alpha, and Simpson's diversity indices for each site ranged from 0.26 to 2.26, from 0.51 to 5.29, and from 0.10 to



0.87, respectively, and were higher at a regional scale for Aichi and Mie (Table 2). Since this was also observed for Fisher's alpha, a diversity index that corrects for sampling intensity, this means that different sites reveal different partners. Sites with multiple ECM tree species (M1 and A1) tended to show higher values (Table 2), but rarefaction analyses in Aichi and Mie at  $n = 11$  showed similar estimated numbers of 21.7 and 17.2 MOTUs, respectively (Table 2 and Fig. S2a). In an NMDS scattering plot, although MOTU assemblages in Aichi and Mie tended to be separated on the first axis, the separation according to regions was not significant (PERMANOVA,  $p = 0.1$ , Fig. S3a). Of the eight variables fitted as vectors to the NMDS plot of the mycorrhizal community of *P. japonica*, PCNM was significantly correlated (Fig. S3b).

### ECM fungal communities around *P. japonica* at Aichi

ECM communities surrounding *P. japonica* were assessed around 6 plants in the Aichi region (four in A1, one in A2 and A3), and 137 sequences of ECM tips were recovered that fell into 33 MOTUs (Table S1) belonging mostly to *Russula* (42.3 %), Helotiales (15.3 %), *Cortinarius* (10.9 %), and Corticiaceae (5.8 %) (Fig. 2b). This is more than the 20 MOTUs found on corresponding *P. japonica*, and indeed diversity indices tended to be higher for the ECM community (Table 2). Although the sampling effort of ECM tips was lower than for *P. japonica* clones, rarefaction in six soil cores of ECM communities at Aichi revealed more MOTUs (15) than did six *P. japonica* plants (five MOTUs; Fig. S4). In addition, the slightly different barcoding method between

**Table 2** Diversity of fungal taxa associated with several *Pyrola* species, including *P. japonica* in this study and surrounding ECM trees

	Examined unit				Diversity indices				Mantle	Molecular typing	References
	Region	Site	Plant/soil core	Root length (mm)	Shannon	Fisher's alpha	Simpson	Richness after rarefaction <sup>d</sup>			
<i>P. japonica</i>	Aichi	A1	5 <sup>a</sup>	60	2.26	3.60	0.87		Absent	Cloning	This study
		A2	3 <sup>a</sup>	36	1.52	1.69	0.75				
		A3	3 <sup>a</sup>	36	0.57	0.51	0.36				
		All	11 <sup>a</sup>	132	2.55	4.60	0.90	21.71			
	Mie	M1	6 <sup>a</sup>	108	1.73	5.29	0.71				
		M2	6 <sup>a</sup>	56	0.34	1.23	0.13				
		M3	8 <sup>a</sup>	78	1.51	3.25	0.70				
		M4	1 <sup>a</sup>	12	0.26	0.97	0.10				
	All		21 <sup>a</sup>	254	1.91	5.44	0.81	17.17			
		Kyoto	K1	3 <sup>a</sup>	36	1.21	1.69	0.64			
All		35 <sup>a</sup>	422	1.55	3.15	0.73	22.51				
	Aichi		6 <sup>b</sup>	140 <sup>c</sup>	2.95	13.80	0.92				
Surrounding ECMs									Direct		
<i>P. chlorantha</i>								Present or absent	Cloning or direct	Tedersoo et al. (2007)	
		Kärle	5 <sup>b</sup>	35 (105) <sup>c</sup>		10.9					
<i>P. rotundifolia</i>								Present	Direct	Vincenot et al. (2008)	
		Kärle	10 <sup>b</sup>	70 (210) <sup>c</sup>	2.37	10.61					
		Värška	10 <sup>b</sup>	70 (210) <sup>c</sup>	2.14	15.65					
<i>P. asarifolia</i>								Present	Direct	Hashimoto et al. (2012)	
		16	31 <sup>b</sup>			17.1	0.96				
		17	16 <sup>b</sup>			6.2	0.84				

<sup>a</sup> Number of plants

<sup>b</sup> Number of soil cores

<sup>c</sup> Number of mycorrhizal roots examined with estimated root lengths in parentheses

<sup>d</sup> Rarefied to a sample number of 11 and Kyoto was not attempted owing to a shortage of plants ( $n = 3$ )

**Fig. 3** Frequencies of mycorrhizal taxa detected from *Pyrola japonica* roots (white square) and surrounding ECM roots (black square) at Aichi. Data were calculated as percentages of the focus taxa within a whole sample ( $n = 369$  clones for *P. japonica* and  $n = 137$  root tips for ECM roots). Asterisks indicate for each MOTU significant differences at  $p < 0.05$  (\*) and  $p < 0.001$  (\*\*\*) of the occurrence frequency in the two communities according to Fisher's exact test

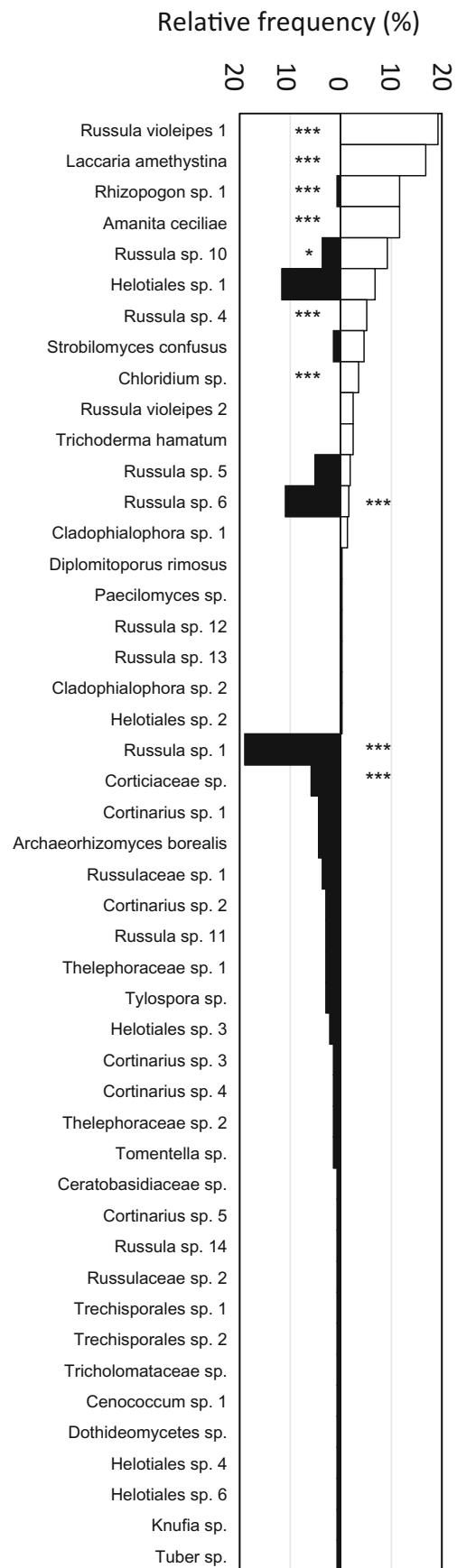
ECM tips and *P. japonica* clones was applied, but the primer LB-W used for the former samples is considered to target basidiomycetes (Tedersoo et al. 2008) and thus valid for the comparison of russulacean fungal occurrences between them. Six out of 47 MOTUs detected in Aichi were shared between *P. japonica* and ECM tips in the same core (Fig. S5): these MOTUs accounted for 35.8 % (132/369 clones) of the *P. japonica* community versus 26.3 % (36/137 sequences) of the ECM tip community (Table S3). Three were *Russula* spp. (spp. 5, 6, and 10), which accounted for 35.6 % (47/132 clones) of the shared community in *P. japonica*; the others were *Rhizopogon* sp. 1, *Strobilomyces confusus*, and *Helotiales* sp. 1, which together accounted for 64.4 % (85/132) of the shared community in *P. japonica* (Tables S2 and S3). Moreover, four taxa found on ECMs were present on *P. japonica* roots from other sites, namely *Russula* spp. 1 and 11, *Cenococcum* sp. 1, and *Helotiales* sp. 4 (Tables S2 and S3). As expected, *Russula* spp. were frequent in the shared community, but strikingly *Russula* sp. 1, which dominated in the Mie region on *P. japonica* (48 % of plants), was absent from *P. japonica* in the Aichi region: this is unexpected because it frequently occurred on ECM tips in Aichi (19 % of ECM tips, in five out of six cores). Yet, total MOTU compositions were significantly different between *P. japonica* and ECM tips at Aichi (Fig. 3;  $\chi^2 = 381.07$ ,  $df = 46$ ,  $p < 0.001$ ).

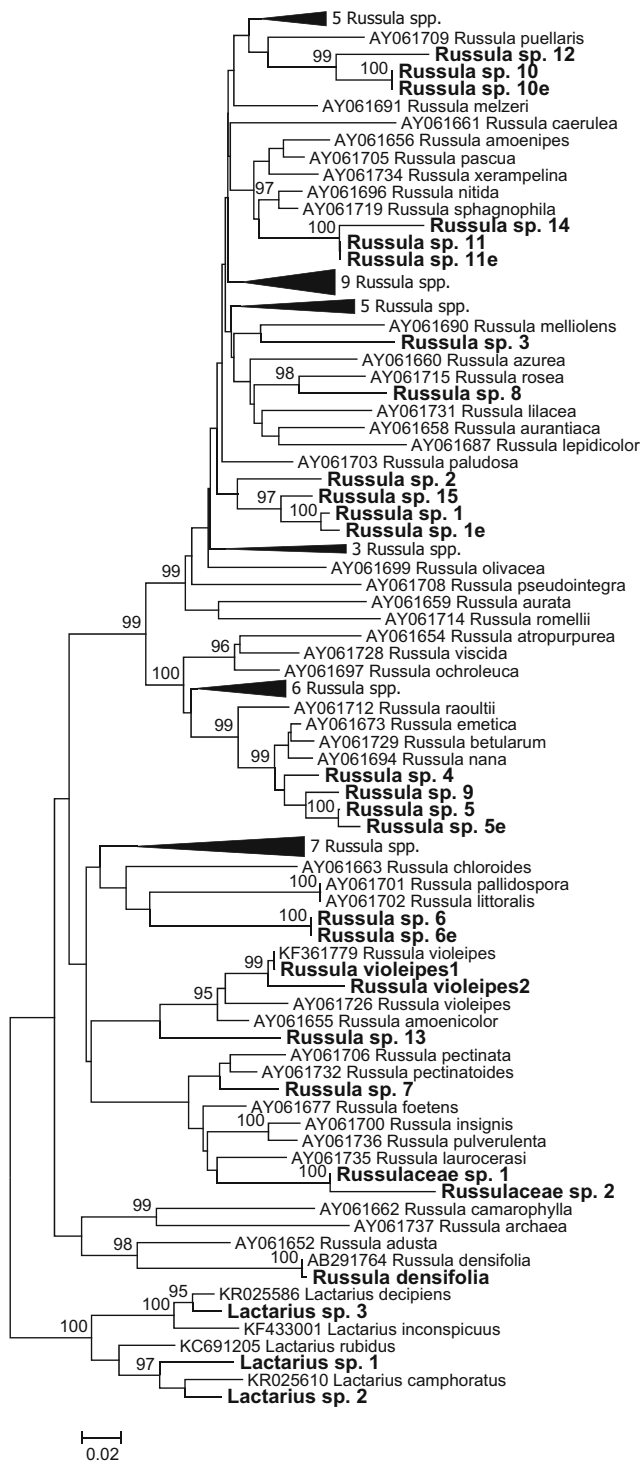
### Phylogenetic positions of russulacean MOTUs

Phylogenetic positions of russulacean MOTUs obtained from *P. japonica* and ECM tips were analyzed using the NJ method (Figs. 4 and S5). Representative sequences of the MOTUs shared between *P. japonica* roots and ECM tips were supported by 100 % of bootstrap values. MOTUs 12 and 15 were confirmed to be *R. densifolia* and *R. violeipes*, respectively. No clear clustering of MOTUs was observed, although some were grouped into close positions, such as *Russula* spp. 4, 5, and 9 and *Russula* spp. 1, 2, and 15.

### Discussion

We analyzed fungal mycorrhizal associates of *P. japonica* grown under various ECM forests of three





**Fig. 4** Neighbor-joining phylogeny with positions of russulacean MOTUs from both *Pyrola japonica* roots and ECM tips recovered in this study (in bold). Bootstrap tests (1000 replicates) larger than 90 % are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method. MOTUs derived from ECM tips are followed by an “e.” Some interior branches were compressed into solid elongated triangles whose thickness was proportional to the number of sequences (an uncompressed tree is to be found in Fig. S5)

sites. Most mycorrhizal fungi belonged to ECM taxa, and 76.2 % of the clones sequenced were affiliated to *Russula* spp., which colonized 94 % of sampled *P. japonica* individuals. In the ECM community studied on tree roots surrounding *P. japonica* at Aichi, *Russula* spp. also dominated. The mycorrhizal community on ECM tips differed significantly from that on *P. japonica* roots, but the different primer sets may cause PCR biases.

#### A preference of *P. japonica* for *Russula* spp.

The predominance of *Russula* spp. was congruent with our previous report (Matsuda et al. 2012) from a single *P. japonica* population (under *Quercus serrata* and *Quercus acutissima* at Tsu City, central Japan; Fig. 1), where 84 % of identified fungi belonged to *Russula*. However, in this previous study, the exclusive use of direct sequencing of fungal ITS discarded many root samples with multiple colonizations: the success rate in Matsuda et al. (2012) was lower (44 % of root fragments) than the current one (85 %). Population origins in central Japan and methodological differences thus had limited impacts on the conclusion that *Russula* spp. dominate among fungi associating with *P. japonica*. Similarly, nearby ECM tree taxa, including *Pinus*, *Castanopsis*, or *Quercus*, did not affect the predominance of russulacean fungi on *P. japonica*. This predominance thus reflects a mycorrhizal preference of this plant for *Russula*. *Russula* spp. from the *delica* group are known to associate specifically with the MX orchid *Limodorum abortivum* (Girlanda et al. 2006; Bellino et al. 2014), and *Russula* spp. are partners to many MH orchids (Merckx 2013). The predominance of russulacean fungi was also reported in the MH Monotropoideae (Ericaceae), a sister group of *Pyrola* plants (Merckx et al. 2013; Matsuda et al. 2011). Although circumstantial evidence has accumulated, it remains unclear why *Russula* spp. support MX and MH plants.

Another unexpected observation is the dominance in some individuals from Aichi and Mie of *L. amethystina*. This Euro-Asiatic taxon (Vincenot et al. 2012) has already been found in European *Pyrola chlorantha* (Tedersoo et al. 2007), as have other *Laccaria* spp. in European *P. minor* (Zimmer et al. 2007) and Japanese *Pyrola asarifolia* (Hashimoto et al. 2012). Yet, *Laccaria* spp. were always a minor component of the diverse communities found on the *Pyrola* host plants. We speculate that the local abundance of *L. amethystina* in our study is relatively boosted by the depauperate fungal community of *P. japonica*, but we cannot exclude a marginal preference for *Laccaria*. Thus, it may be that *Laccaria* acts as a mycorrhizal substitute when the usual dominant fungal partner is missing or no longer available.

Based on our data, *P. japonica* thus displays distinctive features compared with other investigated *Pyrola* spp. that



harbor diverse ECM fungal taxa not only in green, MX, or autotrophic species (Tedersoo et al. 2007; Vincenot et al. 2008; Hynson and Bruns 2009; Toftegaard et al. 2010; Hashimoto et al. 2012) but also in achlorophyllous MH species (Hynson and Bruns 2009). Thus, the genus *Pyrola* is often considered as a generalist lineage in the evolution of mycoheterotrophy (Hynson et al. 2013). Although achlorophyllous MH plant species often show higher mycorrhizal specificity (Merckx 2013), the fully MH *P. aphylla* has a low specificity, comparable to that of the MX *Pyrola picta* (Hynson and Bruns 2009). In the case for *P. japonica*, mycorrhizal fungal richness was far lower than for other *Pyrola* species (consider Fisher's alpha values that are independent of the sampling effort in Table 2), although taxa also reported for other *Pyrola* plants (e.g., *Amanita*, *Laccaria*, Thelephoraceae, and Russulaceae) were marginally involved. Shannon and Simpson diversities were also lower at most sites because evenness was distorted by the dominance of *Russula*.

Habitat characteristics could affect the mycorrhizal community of *P. japonica*. In MH Monotropoideae and MH orchids, surrounding tree species or geography can affect the mycorrhizal community. In the former plants that are phylogenetically close to the genus *Pyrola*, *Monotropa uniflora* and *Pterospora andromedea* associated with different *Russula* (Bidartondo and Bruns 2001) and *Rhizopogon* species (Dowie et al. 2012) depending on the locality, respectively. Similarly, Taylor and Bruns (1999) reported that MH orchids in the genus *Corallorhiza*, growing in either oak- or conifer-dominated forests, associate with different *Russula* communities. In our study, the *P. japonica* mycorrhizal community seems to differ depending on the sites inferred from an NMDS plot, and indeed diversity values are higher at the regional scale than the site scale, suggesting a spatial variation of the fungal associates within Aichi and Mie. Yet, tree taxa were not a significant factor; however, the statistical power of our sampling for this issue was limited (Fig. S3) and the closely located A2 and A3 sites (1.8 km), which differed by tree taxa (Fagaceae versus Pinaceae), had quite different communities. Remarkably, *Russula* sp. 1 was one of the most frequent MOTUs on *P. japonica* at the Mie sites, and among ECM tips at the Aichi sites, but it was never detected from *P. japonica* in the Aichi region (Tables S2 and S3; the proportion of individuals with *Russula* sp. 1 significantly differed between regions  $\chi^2 = 5.56$ ,  $df = 1$ ,  $p < 0.05$ ). Thus, tree taxa as well as geographical locations may influence community patterns of mycorrhizal fungi of *P. japonica*, although this deserves closer investigation. Considering our limited sampling efforts as well as the wide geographical distribution of *P. japonica* in East Asia (Takahashi 1991), further extensive sampling could allow robust investigations of regional and host differences.

To describe all fungal associates, the species accumulation curve should ideally be saturated, but identifying major ECM fungal associates needs only at most several tens of clones or

even direct sequencing (Tedersoo et al. 2007; Kennedy et al. 2011). In this study, we collected up to 64 clones from each plant and that was an effort comparable to the most intense analyses of mycorrhizal fungi associated with MX orchids (Abadie et al. 2006; Giralanda et al. 2006). Although observed MOTU richness does not saturate with 64 clones in most individuals, the diversity index was mostly saturated by the first 20 to 30 clones (Fig. S2b). The Simpson index takes evenness into account so that it rapidly reaches a plateau when one of community member is far more abundant than the others. Thus, even without description of all fungi present in the sample, 20–30 clones efficiently characterized the major partners associated with *P. japonica* and its preferential association with *Russula* spp. In the future, “next-generation” sequencing technologies may provide a more comprehensive picture of *P. japonica* mycorrhizal associations.

### A community filtered from the surrounding ECM community

The ECM communities of trees surrounding six Aichi *P. japonica* plants were also analyzed, and five individuals shared at least one MOTU with neighboring ECM tips from the same core (Table S3). Although shared MOTUs do not necessarily mean a direct mycelial connection with ECM roots, these results are congruent with the presence of common mycorrhizal networks connecting MX plants with surrounding canopy trees (Selosse and Roy 2009; Simard et al. 2012). The existence of a few shared species (12.8 % of MOTUs) that are very abundant (colonizing ca. one third of sampled roots on each partner) is congruent with a previous study of *P. asarifolia* and surrounding trees (Hashimoto et al. 2012) and is also a common trait of many ECM communities in that multihost species are often abundant (e.g., Henry et al. 2015). Moreover, such abundant root colonization may favor abundant mycelial links and thus efficient carbon transfer to the C-receiving MX plant.

Although our analyses of 20–30 ECM tips are likely too limited to provide a comprehensive view of the ECM community at each study site (Miyamoto et al. 2014), the fungal composition significantly differed between *P. japonica* and surrounding trees. For the five most frequent MOTUs on *P. japonica* roots, the relative frequency of russulacean MOTUs was significantly higher than that on ECM tips (Fig. 3), e.g., *R. violeipes* 1 ( $p < 0.001$ ) or *Russula* sp. 10 ( $p < 0.05$ ). Similarly, among the five most frequent MOTUs on ECM tips, the frequencies of *Russula* spp. 1 and 6 were significantly higher ( $p < 0.001$ ) on ECM tips than on *P. japonica*. PCR biases derived from different primer sets used for either *Pyrola* roots or ECM tips may mask the actual differences in ECM fungal assemblages between them. However, the LB-W primer for ECM tips is known to more specifically amplify basidiomycetes (Tedersoo et al. 2008),

and thus any bias might have favored the detection of russulacean groups on ECM tips. The primer change and the use of cloning for *P. japonica* (but not for ECM tips) may have introduced a bias (e.g., cloning could have led to overrepresentation of *Russula* spp. in *P. japonica*), so our comparison is only valid provided that this bias is limited. Under the assumption of a limited bias, the ECM communities were rich in *Russula* spp. (six species representing 42.3 % of the sampled tips), suggesting, as expected, that *Russula*-rich spots may be favorable for *P. japonica* settlement (a similar conclusion was made for the MH *Sarcodes sanguinea* (Ericaceae) and its specific associates *Rhizopogon ellena*; Bidartondo et al. 2000). On the other hand, the absence of some fungi abundant in the surrounding ECM community is significant, and the much lower diversity on *P. japonica* than in the surrounding ECM community suggests that this plant filters the surrounding ECM community. Such filtering, although with much less diversity reduction, may help to explain why the Japanese populations of the related *P. asarifolia* display fungal associates that somehow differ from ECM communities of surrounding trees (Hashimoto et al. 2012).

## Conclusions

The mycorrhizal fungi associated with *P. japonica* confirm our preliminary conclusion (Matsuda et al. 2012) that they are dominated by russulacean fungi, in spite of a diverse local ECM community. This mycorrhizal preference, although not unusual among MX plants, is unique compared with other *Pyrola* spp. Since all study sites were in central Japan, and since we suspect a potential for local variation as discussed above, wider sampling of *P. japonica* covering the natural host distribution would provide more insights into its pattern of fungal preferences.

**Acknowledgments** We thank Mr. S Iio for habitat information regarding the Hashirida shrine, Iyama shrine, and Makiba Mizunosaka West Hill and the Aichi prefecture government office for permission to access the sites. We also thank T. Chikada, Life Science Research Center, Center for Molecular Biology and Genetics, Mie University, for helping out with DNA analyses, the members of the Laboratory of Forest Pathology and Mycology, Graduate School of Bioresources, Mie University, for their help in field sampling, and D. Marsh for correcting our English. This study was partly supported by KAKENHI (22688011, 23658124, 25304026) to YM and the Fondation de France (fond Ars Cuttoli & Appel) to M-AS.

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