Botanica Pacifica. A journal of plant science and conservation. 2018. 7(2): 31–39
DOI: 10.17581/bp.2018.07202

RESEARCH PAPER

Diversity of fungal communities associated with mixotrophic pyroloids (*Pyrola rotundifolia*, *P. media* and *Orthilia secunda*) in their natural habitats

Ekaterina F. Malysheva[1]*, Vera F. Malysheva[1],
Elena Yu. Voronina[2] & Alexander E. Kovalenko[1]

Ekaterina F. Malysheva[1]*
e-mail: e_malysheva@binran.ru

Vera F. Malysheva [1]
e-mail: v_malysheva@binran.ru

Elena Yu. Voronina [2]
e-mail: mvsadnik@list.ru

Alexander E. Kovalenko [1]
e-mail: alkov@binran.ru

[1] Komarov Botanical Institute of the Russian Academy of Sciences, Saint-Petersburg, Russia

[2] Lomonosov Moscow State University, Moscow, Russia

* corresponding author

Manuscript received: 14.05.2018
Review completed: 03.07.2018
Accepted for publication: 11.07.2018
Published online: 13.07.2018

ABSTRACT

As it was shown, mixotrophic plants (MxP) strongly depend on their mycorrhizal fungi for carbon, at least at the early stages of life cycle, and have rather high specificity for mycobionts. However, the diversity of fungi associated with MxP and their role in plant's life are still poorly known, especially under natural conditions. In the present study, the diversity of mycobionts of the three mixotrophic pyroloid species (*Pyrola rotundifolia*, *P. media* and *Orthilia secunda*) was investigated by sequencing *nr*ITS from roots and rhizomes. At the same time, we studied ectomycorrhizal fungal communities of neighboring trees. The mycobiont diversity slightly differed berween the three species, but they also shared similar fungal taxa. The species of basidiomycete genera *Tomentella*, *Piloderma*, *Russula* and *Mycena* were dominant fungal partners of the studied pyroloids. The plants were also colonized by other ectomycorrhizal and saprotrophic basidiomycetes and ascomycetes. The research results showed MxP link to tree species by shared mycobionts and partial mycoheterotrophy by involvement into mycelial network. Thirty nine fungal taxa (at species and genera level) inhabiting pyroloid root system as mycobionts and root endophytes were detected. Their role for plant performance requires further investigation.

Keywords: Pyroleae, arbutoid mycorrhiza, mixotrophy, *nr*ITS, operational taxonomic units, symbionts, endophytes

РЕЗЮМЕ

Малышева Е.Ф., Малышева В.Ф., Воронина Е.Ю., Коваленко А.Е. Разнообразие сообществ грибов, связанных с миксотрофными грушанковыми (*Pyrola rotundifolia*, *P. media* и *Orthilia secunda*) в их естественных местообитаниях. Миксотрофные растения (MxP) сильно зависят в углеродном питании от микоризных грибов, по крайней мере, на ранних стадиях развития, и обладают высокой специфичностью к микобионтам. Однако еще довольно мало известно о разнообразии грибов, связанных с MxP, и их роли в жизни растений, особенно в естественных экосистемах. В настоящей работе было исследовано разнообразие микобионтов трех видов грушанковых с миксотрофным типом питания (*Pyrola rotundifolia*, *P. media* и *Orthilia secunda*) путем выделения ДНК и последующего секвенирования *nr*ITS из корней и корневищ. Одновременно изучался состав грибов, формирующих эктомикоризу с соседними деревьями. Обнаруженные сообщества микобионтов немного отличались у трех видов грушанковых, хотя были выявлены общие симбионты. Доминирующими партнерами в арбутоидной микоризе изученных видов растений явились базидиомицеты из родов *Tomentella*, *Piloderma*, *Russula* и *Mycena*. Растения были также колонизированы другими эктомикоризными и сапротрофными базидиомицетами и аскомицетами. Результаты исследования показали связь MxP с древесными растениями в природе благодаря наличию общих микобионтов, с помощью которых растения вовлекаются в единую мицелиальную сеть. Всего было выявлено 39 грибных таксонов (видового и родового рангов), обитающих в корневой системе грушанковых в качестве микобионтов и корневых эндофитов. Их роль в жизни растений требует дальнейшего изучения.

Ключевые слова: Pyroleae, арбутоидная микориза, миксотрофия, *nr*ITS, операционные таксономические единицы, симбионты, эндофиты

Phylogeny and physiology of pyroloid plants (members of tribe Pyroleae) have attracted great interest from scientists for more than 200 years (Hynson et al. 2009, 2013). One of the problems with the group's taxonomy is the presence of both leafless (potentially mycoheterotrophic plants) and transitional to fully autotrophic forms in the genus *Pyrola* L. As a result of recent studies based on molecular data (Freudenstein 1999, Kron et al. 2002, Liu et al.

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2010), pyroloids have come to be treated as a separate tribe (Pyroleae) in the subfamily Monotropoideae of Ericaceae. One of the essential features of these plants is the production of a vast number of dust-like seeds (the phenomenon of microspermy) with a minimum nutrient supply. It is known that this trait (dust-like seeds) is also typical for many plants, from at least 12 different families including orchids, and has emerged independently in different phylogenetic lines (Eriksson & Kainulainen 2011). Because of limited carbon reserve such seeds evidently strongly depend on the external carbon sources for their germination, and they compensate for this deficiency by symbiotic connections with fungi.

This nutritional mode was termed "initial mycoheterotrophy" (Leake 1994, Merckx 2013). It is noteworthy that all species of *Pyrola* are initially mycoheterotrophic at the early stages of ontogeny but upon reaching full maturity they demonstrate practically the complete range of trophic strategies, from obligate autotrophy to full mycoheterotrophy (e.g. achlorophyllous *Pyrola aphylla* Smith) including transitional forms. The phenomenon of mixotrophy (or partial mycoheterotrophy) is inherent in such transitional forms. It consists in the possibility of simultaneous use of two independent sources of carbon by adult green plants – one as a product of photosynthesis, and another coming through a mycorrhizal association with fungi (Tedersoo et al. 2007, Zimmer et al. 2007, Selosse & Roy 2009, Matsuda et al. 2012). The mixotrophic plants were shown to demonstrate $\delta^{13}C$ and $\delta^{15}N$ values which are the average between these indices typical for fully mycoheterotrophic and autotrophic plants (Hynson et al. 2009). However, the $\delta^{13}C$ value in mixotrophic pyroloids, as in orchids, may depend on the stage of development, type of habitat, lighting conditions and other factors (Bidartondo et al. 2004, Tedersoo et al. 2007, Zimmer et al. 2007, Johansson et al. 2015). Moreover, it was observed that the same *Pyrola* species occurring in different geographic regions significantly vary in the degree of auto- and heterotrophy, and the causes of this variability have not yet been definitively elucidated (Tedersoo et al. 2007, Zimmer et al. 2007, Hynson et al. 2009).

The tribe Pyroleae contains four genera (*Pyrola*, *Orthilia* Raf., *Chimaphila* Pursh and *Moneses* Salisb. ex Gray) and about 40 species of herbs and subshrubs distributed in the Northern Hemisphere that usually grow in forests under the canopy of trees. The pyroloids form an arbutoid mycorrhiza, in which both intracellular and external (mantle) fungal structures are normally formed, and the morphology of the mycorrhizal root tips can be modified, similar to that occurring in ectomycorhizal tree root tips (Robertson & Robertson 1985, Smith & Read 2008). Arbutoid mycorrhiza is poorly known compared to other types of mycorrhizal symbiosis but it is crucial for forest plant communities by providing joint of tree and tree-shrub layers.

The ectomycorrhizal mycobionts can act as fungal partners in for pyroloids thus allowing the formation of mycelial networks linking plants of tree, shrub and herb layers in forest communities (Tedersoo et al. 2007, Zimmer et al. 2007, Massicotte et al. 2008, Vincenot et al. 2008, Hynson & Bruns 2009, Toftegaard et al. 2010, Hashimoto et al. 2012). Such associations have important environmental consequences: pyroloids growing under the forest canopy are provided with additional source of carbon in the form of ectomycorrhizal woody plants` photosynthetic products, but for their part serve as a "depot" of the mycobiont, which is essential when the tree layer is restored after disturbances caused, for example, by clear-cutting (Smith & Read 2008).

Until the present, not much efforts has been devoted to studying the phenomenon of mixotrophy in pyroloids as compared to the orchids (Matsuda et al. 2012). The subject of study was and remains: a study of seed germination physiology; revealing the diversity of fungi forming mycorrhiza with plants at different stages of ontogenesis as well as the specificity of these symbionts for different species from Pyroleae; a study of changes in plant trophic strategies under different environmental conditions. Initially it was supposed, and then there was evidence (Hynson & Bruns 2009, Hashimoto et al. 2012) that all pyroloids (like orchids) are very demanding for the specific composition of the ectomycorrhizal fungi, but, unlike other representatives of Monotropoideae, not throughout the entire life cycle, but only in the early stages of their development. However, the accumulated data is still insufficient to talk about the direct dependence of mixotrophic plant on fungi, even for the most widespread species of pyroloids. There are still a lot of studies to be done in this direction, and the work presented herein is one of them.

The present research addresses the study of mycorrhizal colonization of three pyroloid species (*Pyrola rotundifolia* L., *P. media* Sw. and *Orthilia secunda* (L.) House) widely distributed in coniferous and mixed forests of the European part of Russia. Data on their mycorrhizas are limited and vary considerably depending on the area of study and the type of plant communities.

Several populations of plants growing in different habitats were studied. Identification of fungal mycobionts using molecular techniques was carried out.

MATERIAL AND METHODS

Study site

A total of 67 pyroloid plants at different developmental stages from 16 populations and more than 100 mycorrhizal root tips of surrounding trees were sampled from 5 sites (50×50 m) in European part of Russia in 2016–2017 (from July to September). The studied populations of pyroloid species were at least 10 m apart. All sites are located in temperate climatic zone and represent natural forests or forest plantation. They differ in environmental conditions and plant community composition. Detailed information on the study sites is shown in Table 1.

Sampling procedure and preparation for DNA extraction

At each site, 3–4 plants from each population were randomly selected. Additionally, ectomycorrhizal root tips of surrounding trees were collected in accordance with previously described procedure (Malysheva et al. 2016). The fruitbodies of ectomycorrhizal macromycete species were sampled at the study sites to provide references for spe-

Table 1. Characteristics of study sites.

Study site	Location	Latitude/ longitude	Plant community	Ectomycorrhizal trees in the habitat	Pyroloid species
S1	Moscow Region, Zvenigorod Biological Station, square 6	55°41'28"N, 36°43'06"E	Coniferous forest	*Picea abies, Pinus sylvestris, Betula* sp., *Quercus robur, Sorbus aucuparia, Tilia cordata*	*Pyrola rotundifolia, Pyrola media, Orthilia secunda*
S2	Moscow Region, Zvenigorod Biological Station, square 7	55°41'28"N, 36°43'46"E	Mixed forest	*Betula* sp., *Pinus sylvestris, Quercus robur, Sorbus aucuparia, Tilia cordata, Populus tremula, Salix caprea, Picea abies*	*Pyrola rotundifolia, Pyrola media*
S3	Moscow Region, Zvenigorod Biological Station, square 20	55°41'06"N, 36°43'54"E	Mixed forest	*Picea abies, Pinus sylvestris, Betula* sp., *Quercus robur, Sorbus aucuparia, Populus tremula, Acer platanoides*	*Pyrola media, Orthilia secunda*
S4	Samara Region, vicinities of Pribrezhny	53°29'46"N 49°52'51"E	Pine forest plantation (50–60 years of age)	*Pinus sylvestris, Populus tremula, Tilia cordata*	*Orthilia secunda*
S5	Samara Region, Zhiguli Nature Reserve, vicinities of Gudronny	53°23'15"N 49°45'44"E	Birch forest	*Betula pendula*, *Tilia cordata*, *Sorbus aucuparia, Salix caprea, Acer platanoides, Pinus sylvestris*	*Orthilia secunda*

cies revealed from the root tips examined. The plant root samples with adhering soil were wrapped with polyethylene film or aluminum foil and placed into sealed plastic bags for transporting to the laboratory. During transport and prior to processing, samples were kept moist and stored in the dark at 4°C. In the laboratory, they were thoroughly washed with tap water and processed within 3 days of sampling. Then roots of pyroloids and trees were treated in the same way.

The collected root samples were divided into segments (5–20 mm length). Individual segments of roots and rhizomes that appeared to be colonized by fungi were placed in 2% cetyltrimethylammonium bromide (CTAB) buffer in 1.5 ml tubes for further analysis.

DNA extraction, PCR amplification and sequencing

Fungal DNA was extracted from roots and rhizomes of pyroloids and ectomycorrhizal tips using the Nucleo-Spin Plant II Kit (Macherey-Nagel GmbH & Co. KG), according to the manufacturer's instructions. The fungal *nr*ITS region (ITS1-5.8S-ITS2) was amplified using two sets of primers: ITS1F + ITS4 and ITS1F + ITS4B (Gardes & Bruns 1993, White et al. 1990). Successful amplifications confirmed visually with agarose gel electrophoresis were then cleaned using the GeneJET PCR Purification Kit (Thermo Scientific, Thermo Fisher Scientific Inc., MA, USA) and quantified using the NanoPhotometer P-300 (Implen GmbH, Germany). In the case of two or more bands amplified, the total product was loaded on agarose gel and then each band was extracted from gel and recovered using the GeneJET Gel Extraction Kit (Thermo Scientific, Thermo Fisher Scientific Inc., MA, USA), according to the manufacturer's protocol for gel extraction. The prepared PCR product was then sequenced with the same primer pairs. DNA sequencing was performed on an ABI3130 Genetic Analyzer using BigDye ver. 3.1 chemistry (Applied Biosystems, Foster City, CA, USA). Electrophoretograms were checked using Sequencing Analysis 5.3.1 (Applied Biosystems) and MEGA 6 (Tamura et al. 2013).

All stages of molecular studies were carried out on equipment of the Center for collective use of scientific equipment "Cellular and molecular technology of studying plants and fungi" at the Komarov Botanical Institute of the Russian Academy of Sciences.

Identification of fungal taxa and phylogenetic analysis

Obtained sequences with high quality and degree of similarity (cutoff of 97 %) were clustered into groups and were manually processed and optimized using the MEGA 6. Chimeric sequences assessed by reference-based checking using GenBank were removed from further analyses. We regarded a sequence as a chimera when its ITS1 and ITS2 regions had 98–100 % similarity to different species or >90 % similarity to different genera. The remaining consensus sequences were then analyzed to ascertain taxonomic affinity. Identification of "Operational Taxonomic Units" (OTUs) was based on the BLASTn search algorithm in NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/) or UNITE sequence databases (http://unite.ut.ee/, Kõljalg et al. 2005). The degree of similarity was accepted: for species level ≥97 % (Kõljalg et al. 2013, Smith et al. 2013), for genus level 95–97 % and for family to order level <95 %. When two or more sequences or sequence groups differed to each other but matched the same entry in databases, a number was added to the name to mark the differences (e.g. *Tomentella* sp. 1 and *Tomentella* sp. 2). A list with the taxa names and the accession numbers of the corresponding database entry of the best match is given in Table 2. All newly generated sequences have been deposited in GenBank with corresponding accession numbers.

The fungal sequences generated from pyroloids were used to construct phylogenetic tree. Phylogenetic analysis was carried out separately for Ascomycota and Basidiomycota. The closest-matching sequences from the GenBank and UNITE databases were additionally involved into phylogenetic analyses. For both datasets the sequences were aligned with the Muscle (Edgar 2004) embedded into MEGA 6. The alignments were adjusted manually using the same program. Phylogenetic reconstructions for data sets were performed with maximum likelihood (ML) analyses in the PhyML server, v. 3.0 (http://www.atgc-montpellier.fr/phyml/), with one hundred rapid bootstrap replicates under GTR model.

Table 2. Fungal diversity associated with three pyroloid species (*Pyrola rotundifolia*, *Pyrola media* and *Orthilia secunda*) and surrounding trees at five study sites.

Taxa	Putative ecology	Host plant	Study site	GenBank accession no.	Best BLASTn match (accession no. of GenBank or UNITE)	Similarity (%)
Basidiomycota						
Amanita pantherina	EcM	*Pinus sylvestris*	4	MF926541	*Amanita pantherina* AB080774, AB080776	97
Amphinema byssoides	EcM	*Picea abies*	1	MF92654, MF926543	*Amphinema byssoides* JN943928	99
Bjerkandera adusta	Sap	*P. rotundifolia*	2, 3	MH270625	*Bjerkandera adusta* KC176315	99
Cortinarius cf. *disjungendus*	EcM	*Tilia cordata*	5	MF926544	*Cortinarius disjungendus* KP013191	95
Cortinarius hemitrichus	EcM	*P. media*	2	MH270626	*Cortinarius hemitrichus* DQ097870	100
Echinoderma asperum	Sap	*P. media*	3	MF926545	*Lepiota aspera* AY176354	98
Gerhardtia borealis	Sap	*O. secunda*	1	MH270627	*Gerhardtia borealis* KP858004	99
Hebeloma sp.	EcM	*Populus tremula*	5	MF926546	*Hebeloma subconcolor* KT218391 *Hebeloma leucosarx* KT218361	99
Laccaria proxima	EcM	*Picea abies*	1	MF926547	*Laccaria proxima* KM067833 *Laccaria proxima* JX907813	99
Lactarius cf. *tabidus*	EcM	*Picea abies*	3	MH270628	*Lactarius* cf. *tabidus* LK932113	96
Lactarius torminosus	EcM	*P. media*	2	MH270629	*Lactarius torminosus* DQ367908	98
Lentinus crinitus	Sap	*P. media*, *O. secunda*	3	MF926548	*Lentinus crinitus* GU207289	97
Luellia recondita	PEcM/Sap	*O. secunda*	3	MF926550	*Luellia recondita* JF519370	98
Mycena cf. *cinerella*	Sap	*O. secunda*, *Pinus sylvestris*	1	MF926553, MF926554	*Mycena cinerella* KT900146	98
Mycena cf. *citrinomarginata*	Sap	*P. media*	2	MF926551	*Mycena citrinomarginata* KJ705180	95
Mycena galopus	Sap	*O. secunda*	3	MF926552	*Mycena galopus* KU516420	97
Phanerochaete chrysosporum	Sap	*P. media*	3	MF926555	*Phanerochaete chrysosporium* KM277996	97
Piloderma bicolor	EcM	*P. media*	1	MF926556	*Piloderma bicolor* KP814514	98
Piloderma sphaerosporum	EcM	*O. secunda*, *Picea abies*	1	MF926557, MF926558, MF926559	*Piloderma sphaerosporum* JQ711814	100
Pleurotus ostreatus	Sap	*O. secun*da	3, 5	MF926560	*Pleurotus ostreatus* LC149608	99
Pseudotomentella tristis	EcM	*O. secunda*	3	MH270630	*Pseudotomentella tristis* KY681465	99
Russula aeruginea	EcM	*Pinus sylvestris*	5	MF926562	*Russula aeruginea* KX095030	97
Russula consobrina	EcM	*P. rotundifolia*	1	MF926563	*Russula consobrina* JF908696	97
Russula font-queri	EcM	*Pinus sylvestris*		MF926561	*Russula font-queri* KT934003, KU949378	99
Russula intermedia	EcM	*Picea abies*	3	MH270631	*Russula intermedia* UDB019791	98
Russula laricina	EcM	*Pinus sylvestris*	4	MF926568	*Russula laricina* KF850405	100
Russula cf. *subfoetens*	EcM	*P. rotundifolia*	2	MH270632	*Russula* cf. *subfoetens* KF245511	98
Russula velenovskyi	EcM	*O. secunda*, *Tilia cordata*	5	MF926564	*Russula velenovskyi* JQ888202	99
Russula cf. *versicolor*	EcM	*Betula pendula*	5	MF926565, MF926566	*Russula versicolor* JQ711937	99
Russula sp.	EcM	*Pinus sylvestris*	4	MF926567	*Russula laricina* KF850405	95
Tomentella stuposa	EcM	*Picea abies*, *O. secunda*	1	MF926571, MH270637	*Tomentella stuposa* JQ888213	99
Tomentella sp.1	EcM	*O. secunda*	1	MF926573	*Tomentella* sp. AJ534915	99
Tomentella sp.2	EcM	*P. rotundifolia*, *O. secunda*	2, 3	MF926574, MF926575	*Tomentella sublilacina* HM189985	95
Tomentella sp.3	EcM	*P. rotundifolia*	1	MH270633	*Tomentella* sp. JQ711817	98
Thelephora cf. *anthocephala*	EcM	*O. secunda*, *Populus tremula*	5	MF926569, MF926570	*Thelephora anthocephala* KP454019	95
Tylospora fibrillosa	EcM	*O. secunda*	3	MF926576	*Tylospora fibrillosa* JQ711996	98
Ascomycota						
Acremonium sp.	Sap	*P. rotundifolia*	1	MF926521	*Acremonium* sp. KU376503, LT598644	97
Aureobasidium sp.	Sap	*O. secunda*	4	MF926522	*Aureobasidium pullulans* KX067792, KX444670	90
Cadophora finlandica	Endo, PEcM, Eric	*P. media*	3	MF926523	*Cadophora finlandica* EF093179	97
Chrysomyxa pyrolae	Pathog	*P. media*	3	MF926524	*Chrysomyxa pyrolae* GU049485	99
Cladosporium cladosporoides	Sap	*P. rotundifolia*	2	MH270634	*Cladosporium cladosporoides* MF077224	98
Helvella sp.	EcM	*O. secunda*	5	MF926525	*Helvella* sp. KM576410	99
Humaria hemisphaerica	EcM	*O. secunda*	1, 4	MF926526, MF926527, MF926528	*Humaria hemisphaerica* DQ200832	98
Ilyonectria sp.	Pathog	*P. rotundifolia*	3	MH270635	*Ilyonectria* sp. KU556513	100
Oidiodendron maius	Eric	*O. secunda*	4	MF926533, MF926534	*Oidiodendron maius* AF062800, AB089654	97
Pezoloma ericae	Eric	*Pinus sylvestris*	1	MF926532	*Rhizoscyphus ericae* JQ711893	97
Phialocephala europaea	Endo	*O. secunda*	4	MF926529	*Phialocephala europaea* AY078137	99
Phialocephala fortinii	Endo	*O. secunda*, *Picea abies*	4, 1	MF926530, MF926531	*Phialocephala fortinii* KX440138	98
Sphaerosporella sp.	PEcM	*O. secunda*	1	MH270636	*Sphaerosporella* sp. JQ711781	97
Thuemenidium atropurpureum	Sap/ Unknown	*O. secunda*, *P. media*	4, 1, 3	MF926535, MF926536, MF926537, MF926538, MF926539	*Thuemenidium atropurpureum* JQ256427	99
Tuber pacificum	EcM	*O. secunda*	5	MF926540	*Tuber pacificum* JQ712002	98
Wilcoxina rehmii	EEnM	*O. secunda*	3, 4, 5	MF926517, MF926518, MF926519, MF926520	UNITE Species Hypotheses *Wilcoxina rehmii* JQ975970	99

EcM – ectomycorrhizal, EEnM – ectendomycorrhizal, PEcM – putative ectomycorrhizal, Eric – ericoid mycorrhizal, Sap – saprotrophic, Endo – endophytic, Pathog – pathogenic, Unknown – with unknown trophic status

Determination of OTUs trophic status

The putative trophic status of the detected fungal OTUs was assigned based on a critical review of available literature (Massicotte et al. 1993, Erland 1995, Rice & Currah 2006, Gorfer et al. 2007, Tedersoo et al. 2007, 2010).

RESULTS

In total, 220 fungal DNA samples were obtained from the roots and rhizomes of studied pyroloid plants and 110 from the ectomycorrhizal root tips of surrounding trees. From pyroloids only 120 fungal *nr*ITS sequences were generated and from trees – 45 sequences. In our work we obtained a rather large number of heterogeneous PCR products (not included in subsequent analysis), that may be due to prevailing colonization of pyroloid's roots with numerous endophytes together with mycorrhizal symbionts, what was also noted for Pyroleae by other authors (Tedersoo et al. 2007, Vincenot et al. 2008). Overall, 52 OTUs of species or generic level were detected based on best BLASTn matches. The taxonomic hypotheses and putative ecology of revealed OTUs are provided in Table 2.

The ecology of recovered taxa associated with pyroloids did not significantly differ between the three species. Most of them represented ectomycorrhizal fungi (EcM, 17 OTUs), other were putative ectomycorrhizal (PEcM) or saprotrophic fungi (Sap, 17 OTUs), ectendomycorrhizal fungi (EEnM, 1 OTU), ericoid mycorrhizal fungi (Eric, 2 OTUs), and endophytes (Endo, 2 OTUs). The remaining taxa represented pathogenic fungi numbered 2 OTUs.

It is noticeable that most of the identified fungi belong to taxa from Basidiomycota (27 OTUs or 51.9 % of total diversity) and Ascomycota (5 OTUs or 9.6 %) known as active participants of ectomycorrhizas with trees. At the family level following mycorrhizal and PEcM/saprotrophic fungi from the roots and rhizomes of the three species were identified: Thelephoraceae, Atheliaceae, Mycenaceae, Russulaceae, Phanerochaetaceae, Polyporaceae, Hydnodontaceae, Meruliaceae, Agaricaceae, Cortinariaceae, Pleurotaceae, Lyophyllaceae of Basidiomycota, and Pyronemataceae, Myxotrichaceae, Geoglossaceae, Saccotheciaceae, Tuberaceae, Helvellaceae, Vibrisseaceae, Cladosporiaceae of Ascomycota (and two representatives of Hypocreales and Helotiales with uncertain family affiliation) (Fig. 1).

Owing to their potentially pathogenic, endophytic or contaminant nature, the following taxa were not treated as mycorrhizal or putative mycorrhizal fungi and excluded from the phylogenetic analysis (Fig. 2): *Chrysomyxa pyrolae*, *Ilyonectria* sp., *Acremonium* sp. and *Cladosporium* sp.

The richest in the number of species were corticioid genera *Tomentella* and *Piloderma*, as well as agaricoid genera *Russula* and *Mycena*, which were commonly found in roots of both pyroloid plants and neighboring coniferous trees (mostly in *Picea* and *Pinus* roots). The fungal partner *Tomentella* sp. 2 was shared by *Orthilia secunda* and *Pyrola rotundifolia*, whereas *Thuemenidium atropurpureum*, *Wilcoxina rehmii* and *Lentinus crinitus* were common to *Orthilia secunda* and *Pyrola media* (Fig. 2, 3). The mycobionts *Piloderma sphaerosporum*, *Tylospora fibrillosa*, *Russula velenovskyi*, *Tomentella* sp. 1, *Pseudotomentella tristis*, *Thelephora* cf. *anthocephala*, *Luellia recondita*, *Lentinus crinitus*, *Mycena* cf. *cinerella*, *Mycena galopus*, *Pleurotus ostreatus*, *Gerhardtia borealis*, *Humaria hemisphaerica*, *Phialocephala europaea*, *P. fortinii*, *Oidiodendron maius*, *Sphaerosporella* sp., *Aureobasidium* sp., *Tuber pacificum* and *Helvella* sp. were associated only with *Orthilia secunda*. The following fungi: *Piloderma bicolor*, *Phanerochaete chrysosporium*, *Cortinarius hemitrichus*, *Lactarius torminosus*, *Echinoderma asperum*, *Mycena* cf. *citrinimarginata*, *Cadophora finlandica* and *Chrysomyxa pyrolae* were associated with *Pyrola media*, but *Russula consobrina*, *R. subfoetens*, *Tomentella* sp.3, *Bjerkandera adusta*, *Ilyonectria* sp., *Acremonium* sp. and *Cladosporium* sp. were detected from *Pyrola rotundifolia* root system.

DISCUSSION

Molecular identification of fungi in the selected root fragments from *Pyrola rotundifolia*, *P. media* and *Orthilia secunda* revealed a broad range of fungal taxa (39 OTUs). Among the mycobionts associated with the three species the group of Basidiomycota dominated and numbered 24 OTUs (62 %), followed by Ascomycota with 15 OTUs (38 %). The vast majority of OTUs detected belonged to EcM fungi. They are mostly represented by members of genera *Tomentella*, *Thelephora*, *Piloderma*, *Russula*, *Lactarius*, *Cortinarius*, *Hebeloma*, *Tuber*, *Wilcoxina* and others. Basidiomycetes *Piloderma bicolor* and *P. sphaerosporum* as well as ascomycetes from genera *Helvella*, *Tuber* and *Wilcoxina* are well known as EcM fungi, which confirms the possibility of carbon inflow to the pyroloids through the fungal symbionts

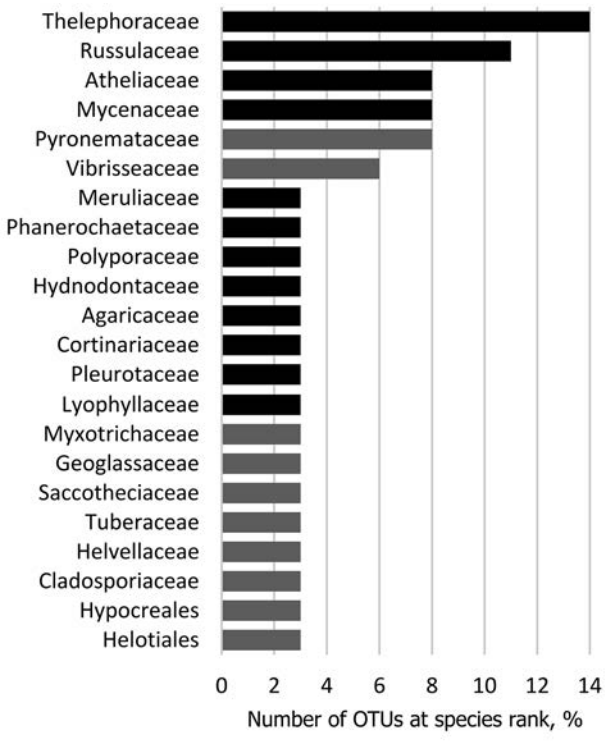

Figure 1 Relative abundance at family (and order) rank of ectomycorrhizal, putative ectomycorrhizal and saprotrophic fungi associated with three pyroloid species (*Orthilia secunda*, *Pyrola rotundifolia* and *P. media*). Black colour indicates basidiomycetes taxa, gray – ascomycetes taxa

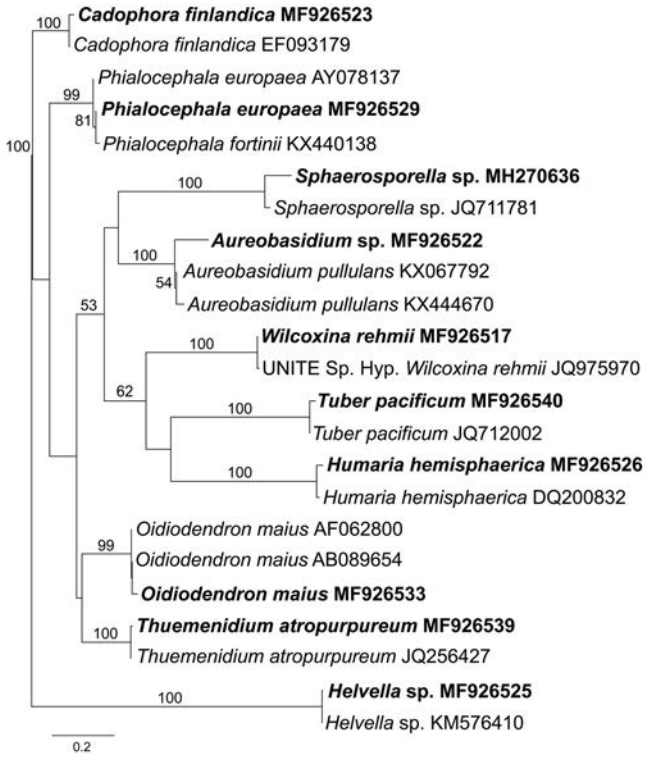

Figure 2 Phylogenetic placement of ascomycetes mycobionts from *Pyrola rotundifolia*, *P. media* and *Orthilia secunda* root systems detected in this study. Newly generated *nr*ITS sequences (indicated by bold font) were complemented with reference sequences derived from GenBank and analyzed with maximum likelihood using PhyML. The tree is midpoint rooted. Branch support is given above the branches

common with photosynthetic wood plants (Rinaldi et al. 2008). The presence of mutual fungal partners was confirmed using molecular method in our study. The dominant mycobionts shared by both pyroloids and surrounding trees in three sites were: *Piloderma sphaerosporum*, *Tomentella stuposa* isolated from *Orthilia secunda* and *Picea abies* roots in S1, *Russula velenovskyi* – from *O. secunda* and *Tilia cordata*, *Thelephora* cf. *anthocephala* – from *O. secunda* and *Populus tremula* in S5, and *Phialocephala fortinii* – from *O. secunda* and *Picea abies* in S1 and S4. The fungal symbionts in these ternary functional complexes "mixotrophic plant-EcM fungus-woody plants" are for the most part not strictly specialized for a particular tree species. Therefore, it is apparent that the adult pyroloid plants are able to use a rather wide range of tree partners as their heterotrophic carbon source.

Most of the taxa revealed in our work as symbionts of the studied plants encompassed those already identified for other pyroloids (*Pyrola* and *Orthilia*) in Europe and North America, corroborating data from similar studies (Tedersoo et al. 2007, Zimmer et al. 2007, Massicotte et al. 2008, Vincenot et al. 2008, Hynson & Bruns 2009, Toftegaard et al. 2010). This is the evidence for the pyroloid species tendency to associate with similar fungal taxa, regardless of plants` geographical distribution. However, a large group of EcM fungi from the order Sebacinales forming mycorrhiza with pyroloids at the early (heterotrophic) stage of their development (Hynson et al. 2013), was not detected in our study.

Although mycorrhizal fungal symbionts play a crucial role in the pyroloids performance, precious few is known about their interactions with potentially diverse communities of so-called root-associated fungi (RAF) or endophytes. At present, evidence is beginning to accumulate that they may act as EcM fungi or partners in ericoid mycorrhizas (Rinaldi et al. 2008). We found three OTUs belonging to RAF community, among them were *Phialocephala fortinii* and *P. europaea* capable of forming stable but nonspecific connections with roots of many plant species (Queloz et al. 2005). The taxa detected from *Pyrola media* root system also included a provisional endophyte, *Cadophora finlandica*, whose ability to form ericoid mycorrhiza and ectomycorrhiza has been reported previously in several studies (Gorfer et al. 2007, Peterson et al. 2008).

For some fungal taxa identified, the association with the roots of pyroloids has been revealed for the first time: ascomycetes – *Phialocephala europaea*, *Oidiodendron maius*, *Thuemenidium atropurpureum*, *Aureobasidium* sp., *Tuber pacificum*, *Sphaerosporella* sp. and *Helvella* sp., and basidiomycetes – *Bjerkandera adusta*, *Cortinarius hemitrichus*, *Echinoderma asperum*, *Gerhardtia borealis*, *Lactarius torminosus*, *Lentinus crinitus*, *Luellia recondita*, *Mycena galopus*, *M.* cf. *cinerella*, *M.* cf. *citrinomarginata*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Pseudotomentella tristis*, *Russula* cf. *subfoetens*, *Tylospora fibrillosa*. For some of them, the ecological role is quite apparent (for example, for *Cortinarius*, *Russula*, *Lactarius*, *Tuber*, *Helvella*, *Tylospora*, which are typical EcM fungi), but a function of other taxa in the plant community remains a mystery. In the present study, we can only register, for the time being, the fact of their presence in the roots of the studied plants and hypothesize their involving into the arbutoid mycorrhiza forming. To make more reliable conclusions about their relationship with plants, more research is necessary.

The most common species *Oidiodendron maius*, a well-known symbiont in ericoid mycorrhizas (Read 1996), identified in the present study, was only once detected from pyroloids roots (*Pyrola morrisonensis*, Matsuda et al. 2017). It has to be born in mind that, as yet, we have no clear explanation for the nature of the relationship between symbionts and hosts in ericoid mycorrhizas (Rice & Currah 2006): whether this is true mutualistic interrelation, or the plant acts as a preventive refugia for fungus, or fungus parasitizes the plant, or there is a certain combination of those three states. *In vitro* experiments display that *O. maius* can improve the growth of host plants by facilitating their nutrition (preferably nitrogen) and detoxifying the soil environment (Rice & Currah 2006, Daghino et al. 2016), though the feedback benefit for *O. maius* is not so obvious. This fungal species possesses prominent enzyme systems (Wei et al. 2016) and can decompose complex polymer compounds in soil, therefore it is highly unlikely that the fungus rely entirely on its photosynthetic host for surviving. Nevertheless, the fungus may obtain some products of photosynthesis in addition to saprotrophic carbon, that potentially gives *O. maius* a competitive advantage over other soil-inhabiting fungi. The question of the advantages for both partners under particular environmental conditions that determine the functional nature of these relations is still open. The tendency to microspermy

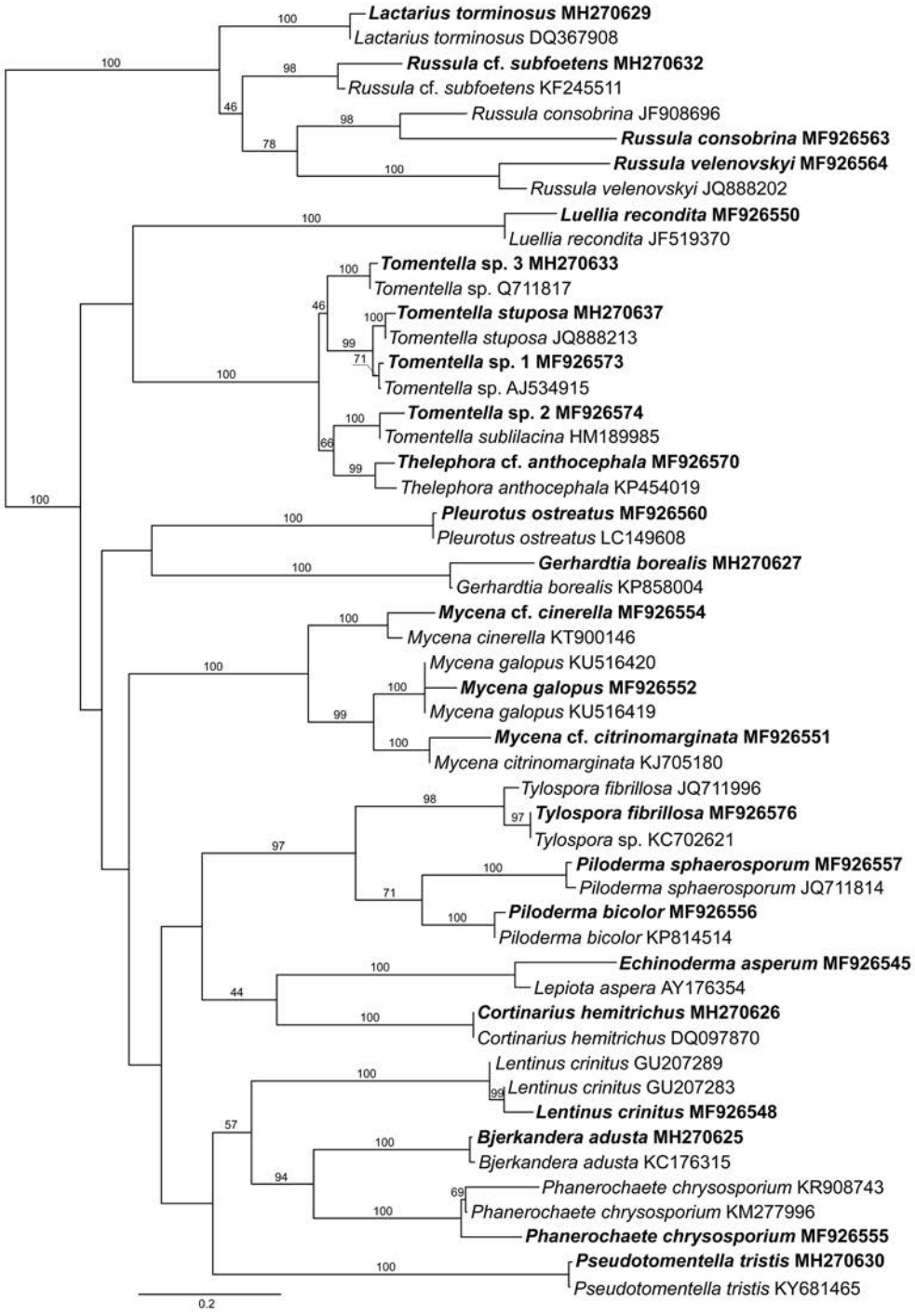

Figure 2 Phylogenetic placement of basidiomycetes mycobionts from *Pyrola rotundifolia*, *P. media* and *Orthilia secunda* root systems detected in this study. Newly generated *nr*ITS sequences (indicated by bold font) were complemented with reference sequences derived from GenBank and analyzed with maximum likelihood using PhyML. The tree is midpoint rooted. Branch support is given above the branches

in Ericaceae as well as saprotrophic ability of its fungal partners (defined as endophytes or symbionts) indicate that ericoid mycorrhizal associations may represent another example of fungal parasitism controlled by host plant similar to one observed in orchids. Such plants taking *O. maius* into their functional area gain a competitive advantage in plant community by increasing supply of organic matter inaccessible to other plants. Given the wide taxonomic tolerance that ericoids show towards endophytic fungi *in vitro* (Rice & Currah 2006), there is an obvious need for detailed examination of endophytic fungi diversity *in situ* and enigmatic ecological role of Myxotrichaceae, Helotiales and close taxa.

A very interesting result of our work is a repeated discovery (in six samples) of *Thuemenidium atropurpureum* from roots of *Pyrola media* and *Orthilia secunda* in three study sites (S1, S3, S4). This species from the family Geoglossaceae s.l. is usually regarded as typical inhabitant of pastures or meadows on sandy or acidic soils, and rarely occurs in woodland (Kučera et al. 2008). Up to now, no obvious ecological connection of this species with any groups of plants was

observed, and its function in plant communities was not clarified. Alternatively, it is known that the closest species, *Sabuloglossum arenarium*, growing under similar ecological conditions, is associated with plants from Empetraceae and Ericaceae (Ericales) (Beenken & Horn 2008). To our knowledge, one of the characteristics of fungi that form eryticoid mycorrhizas is their ability to enhance the availability of N for plants (Read 1996). Most plants can consume nitrogen in two forms – nitrate (NO_3^-) or ammonium (NH_4^+). Some laboratory experiments (Rosen et al. 1990, Smith & Read 2008) demonstrated that many members of Ericales are not capable of consuming NO_3^- as a source of nitrogen. This is accounted for by the adaptation of given plants to acidic soil habitats (with pH in the range 4–5.5), where the nitrate form of N is poorly represented and therefore not available to plants. Recent works (Kosola et al. 2007, Yin et al. 2010) showed that an inoculation of ericoids with appropriate symbiotic fungi (e.g. *Pezoloma ericae*) significantly increases the ability of plants to adsorbtion of nitrogen in the form of nitrates and provides an advantage in growth over other competitors in certain environmental conditions. This provides the opportunity to propose that, in our case, there is a similar situation observed, when *T. atropurpureum* may play an important role in helping the pyroloids to use more effectively organic and mineral resources from strongly acidic soils under coniferous (*Picea* and *Pinus*) forests.

In the roots and rhizomes of the studied model species, we found quite a lot of saprotrophic basidiomycetes (from genera *Bjerkandera*, *Gerhardtia*, *Luellia*, *Phanerochaete*, *Lentinus*, *Echinoderma* and *Pleurotus*). All species from the genus *Mycena* were commonly interpreted previously as saprotrophs, though some were also registered occasionally as mycobionts in *Pyrola media* roots (Toftegaard et al. 2010) or as symbionts of orchid mycorrhizas (Ogura-Tsujita et al. 2009, Hashimoto et al. 2012). This fact may indicate that there is a general mixotrophic strategy for green orchids and pyroloids with similar suite of fungal symbionts, although the connection with certain taxa of fungi is more obligate for orchids. Despite free-living saprotrophs can have part in forming orchid mycorrhizas, it is most likely that the fungi we revealed in the present study may represent potential partners of ectomycorrhizas, and a closer examination of the surrounding trees roots is required.

Future research, no doubt, will clarify the ecological role of many fungi presently detected as well as their relations with pyroloid plants.

ACKNOWLEDGEMENTS

The authors are sincerely grateful to Zhiguli Nature Reserve and Zvenigorod Biological Station staff for their assistance in field research. The research was financially supported by the Russian Foundation for Basic Researches, project No. 16-04-01468 (to E. Malysheva, V. Malysheva, E. Voronina, A. Kovalenko – probes sampling, molecular research). Partial financial support by the Russian Science Foundation (RSCF), programme 14-50-00029, to E. Voronina (macrofungal species morphological identification and herbarization) is acknowledged.

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