Ceratobasidioid mycobionts in Russian populations of Goodyera repens and mycorrhizal specificity in Goodyerinae subtribe (Orchidaceae)

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Abstract

Members of Ceratobasidiaceae family (more commonly known by its anamorphic name “rhizoctonias”) possess a variety of nutritional modes: plant pathogens, saprotrophs, endophytes and symbionts of orchid mycorrhiza. Links between nutritional modes and taxonomy of these fungi as well as their specificity towards plant host is still ambiguous. The scope of the present study was to explore biodiversity of ceratobasidioid mycobionts of sciophytic terrestrial orchid *Goodyera repens*, search for evolutionary stable clades within mycobionts of Goodyerinae subtribe uniform by plant host or geographic region and to establish possible connection between ceratobasidioid nutritional modes and morphological characteristics. We consider *G. repens* a generalist associated with a wide range of distantly related mycobionts. Two unidentified *Ceratobasidium* species and *Thanatephorus ochraceus* are reported from *G. repens* roots for the first time. Ceratobasidiaceae tend to form stable clades specific to either temperate or tropical region. Morphological characteristics of pathogenic and mycorrhizal rhizoctonia isolates tend to form a variety of transitional forms to correlate with nutritional mode.

Introduction

Orchid mycorrhiza (OM) is the recently emerged type of symbiosis: molecular clock approach estimates its repeated origins as the middle Cretaceous (Strullu-Derrien et al. 2018). Due to comparatively brief history of coevolution with orchids, symbiotic fungi may not be taxonomically detached from non-mycorrhizal ones. Even though OM is formed only by members of Orchidaceae, the presence of evolutionary stable clades of specifically OM fungi is still ambiguous. There are numerous examples of OM fungal symbionts with more than one nutritional modes at a time. Thus, OM fungal assemblage can embrace free-living wood-decayers and soil saprobes along with host-dependent ectomycorrhizal or non-orchid plant pathogenic species (Smith and Read 2008).

Anamorphic rhizoctonias (former genus *Rhizoctonia* species, initially not ascribed to any basidiomycete teleomorphs) were the first OM symbionts revealed by means of plant root tissue microscopy and regular isolations in pure culture with subsequent reinoculation (Bernard 1904; Fuller 1909). For a long time they were regarded as the only symbionts in OM, and even after the other groups of symbiotic fungi were detected in orchid roots, the green orchids considered to be associated largely with rhizoctonias contrary to achlorophyllous species recruiting diverse saprotrophic and ectomycorrhizal macrofungi (for more details see Smith and Read 2008). The data obtained during the last two decades allow to consider the more complicated orchid-rhizoctonia interactions, for the latter turn out to be rather common mycobionts for not only green but non-photosynthetic plant species too (e.g. Bougoure et al. 2009; Pecoraro et al. 2020).

Despite of the fact that OM research pioneered by Noël Bernard (Selosse et al. 2011) was originated with observation of symbiotic rhizoctonias, its taxonomic status remained obscure for a quite long period. The first evidence of the genus polyphyly was provided by Warcup and Talbot (1967) who successfully obtained teleomorphic stages under cultural condition. The more recent studies applying molecular techniques resulted in consistent conclusions, and current view of rhizoctonias implies a form complex placed within basidiomycete orders Cantharellales (Ceratobasidiaceae, Tulasnellaceae) and Sebacinales (Sebacinaceae) (Smith and Read 2008 and references therein; Pilshchikova and Gannibal 2016; Weiß et al. 2016; Oberwinkler et al. 2017). Both taxa mentioned are known for broad mycorrhizal activity.

Rhizoctonial mycobionts of Ceratobasidiaceae affinity require particular attention due to variety of nutritional strategies presented (economically important plant pathogens, saprotrophs, endophytes and OM symbionts), a number of yet unresolved questions concerning its evolution and specificity, and controversy of published data on correlation between nutritional mode and morphological and genetic traits (Veldre et al. 2013).

Due to poor cultural morphology of Ceratobasidiaceae various characteristics were tested as diagnostic to build phylogeny and describe new taxa. Morphology of basidia was used to describe genera (Tu and Kimbrough 1978), while indirect traits were used on species level such as plant host (Constantin and Dufour 1920), anastomosis groups and nuclear status (Pilshchikova and Gannibal 2016). The two latter characteristics are shown to correlate with ecological status and ITS phylogeny: multinucleate isolates were described as plant pathogens, whereas binucleate isolates may appear as both pathogens and OM mycobionts (Kataria, Hoffman, 1988; Soelistijono et al. 2020). However, these traits are not useful to reconsider existing phylogeny of this family. Therefore, due to uncertain phylogeny, poor morphology and lack of alternative phylogenetic markers in majority of studies Ceratobasidiaceae fungi are not identified to species level.

Currently near all previously described ceratobasidioid species are placed within either *Ceratobasidium* or *Thanatephorus* (MycoBank 2023). These genera represent a wide range of fungal life styles, both saprotrophic and biotrophic. Extensive attempts to build a family-scale phylogeny show that ITS phylogeny of Ceratobasidiaceae partly correlate with nutritional mode, anastomosis group and nuclear status of isolates. However, to build highly supported phylogeny ITS region should be supplemented with additional phylogenetic and morphological markers (Oberwinkler et al. 2013; Veldre et al. 2013).

Therefore, current knowledge on Ceratobasidiaceae taxonomy allows us to investigate specificity of OM by searching of highly supported taxonomic clades that are specific for certain plant host or geographic region which may be further described as new species. This approach may reveal evolutionary traits of mycorrhizal Ceratobasidiaceae. Most studies show that each orchid individual possesses only one mycobiont, but at plant species level there are evidences of a wide range of mycobionts existence (Yagame et al. 2008; Shefferson et al. 2015; Rammitsu et al. 2019). Therefore, the current study aims on the biodiversity of ceratobasidioid orchid symbionts study with the example of globally distributed Goodyerinae subtribe with emphasis on model plant species *Goodyera repens* (L.) R.Br. in W.T.Aiton; on the search for Ceratobasidiaceae clades specific for certain plant host or geographic region; and on providing characteristics of fungal isolates that could shed light on nutrition modes evolution in Ceratobasidiaceae.

Materials and methods

Studied plants description

Subtribe Goodyerinae (Cranichidae, Orchidoideae) has worldwide distribution and includes approximately 27 genera of terrestrial sciophytic rhizome-forming orchids (Chen et al. 2019). The most species-rich genera are *Goodyera* inhabiting mostly temperate Europe, Asia and Northern America (Kallunki 1976) and *Anoectochilus* predominantly inhabiting tropical Asia and America (Zettler et al. 2012; Bon et al. 2020).

Goodyera repens is a rhizome-reproducing clonal orchid that inhabits conifer forests in temperate regions of the northern hemisphere (Łazarski 2021). This species may be regarded as a model object in OM studies as it was used in the experiments of Cameron et al. (2006) to prove mutualistic nature of OM. Isotopic evidences assume autotrophy of adult *G. repens* individuals, but further research is required (Hynson et al. 2009; Voronina et al. 2018).

Dominant mycobionts associated with *G. repens* are members of Ceratobasidiaceae, namely *Ceratobasidium cornigerum* (Bourdot) D.P. Rogers, originally described as *Rhizoctonia goodyerae-repentis* Costantin & L.M. Dufour (Constantin and Dufour 1920; Cameron et al. 2006). This species is binucleate and regarded as hemi-biotroph possessing the ability to exist as both plant pathogen and OM symbiont (Newton et al. 2010). Moreover, presence of ectomycorrhizal fungi in *G. repens* roots may indicate the ability of this species to form mycorrhizal networks with conifer trees (Voronina et al. 2018).

Sampling sites and sample collection

The material used for the study were 11 samples of *G. repens* roots and 11 matching soil samples at the root depth within 10 cm range from corresponding plants. The sampling was conducted in 2021 and 2022 on 11 sites located in 3 regions of European part of Russia: Leningrad region (2 sites in surroundings of Bolshoe Lesnoe lake, Vyborg district), Moscow region (3 sites on the territory of Moscow State University Zvenigorod biological station), and Karachay-Cherkessia republic (6 sites in Teberdinskiy natural reserve). All sampling sites were located in conifer forests predominated by *Pinus sylvestris* and *Picea abies* in Leningrad and Moscow regions and by *P. sylvestris, P. abies*, and *Abies nordmanniana* in Karachay-Cherkessia. Sites L1 and L2 were distant from conifer trees, T4 was located on a granite stone with scarce soil covering with no access for conifer roots. Altitude of Karachay-Cherkessia sites varied from 1675 to 1987 m above sea level. The short description of sampling sites is summarized in Table 1.

Samples were stored under 4^{o}C in absolute ethanol for metagenome analysis and in paper bags for culture isolation.

Table 1
Description of sampling sites

Region	Site	Coordinates	Nearby conifers	Altitude, m
Leningrad region	L1	60.800425, 28.941970	None	ND*
	L2	60.801061, 28.950757		ND
Moscow region	M1	55.691680; 36.715776	*Pinus sylvestris, Picea abies*	ND
	M2	55.691485; 36.714948	*P. abies*	ND
	M3	55.694775; 36.739656	*P. sylvestris, P. abies*	ND
Karachay-Cherkessia	T1	43.446608, 41.704962	*P. sylvestris*	1987
	T2	43.441927, 41.704291		1982
	T3	43.438027, 41.703754		1960
	T4	43.437247, 41.706977	None	1763
	T5	43.437539, 41.709663	*Abies nordmanniana*	1685
	T6	43.438027, 41.713557	*P. abies*	1675

*ND – not defined

Isolation and identification of fungal cultures

Fungal cultures were isolated from root fragments of approximately 1 cm long cleared from debris and soil residues, sterilized by placing in 70% ethanol, amoxicillin solution, surfactant and rinsed in sterile distilled water. Sterilized root fragments were placed on Petri dishes with malt extract agar and cultivated under 26°C for 7 days.

Fungal isolates were identified by sequencing of ITS region. Genomic DNA was isolated using DNA extraction kit, amplified using ITS1 and ITS4 primers (chemicals and primers are provided by Evrogen Co, Russia) and sequenced by Evrogen Co.

Metagenome analysis

The samples were processed the same way as for culture isolation followed by disintegrating in mortar. Genomic DNA was isolated using FastDNA SPIN Kit (MP Biomedicals, USA), fungal ITS2 regions were amplified using NR_5.8SR and NR_ITS4R primers (Evrogen Co, Russia). Next generation sequencing was performed by BioSpark Co (Russia) on Illumina MiSeq sequencer (Illumina, USA) with generation of 5000 reads per sample. Sequences were processed with QIIME 1.9.1 algorithm. Analysis was made by BioSpark Co (Russia).

Fluorescence microscopy

Fluorescence microscopy was used for visualization of nuclei in cells of Ceratobasidiaceae cultures and pelotons in *G. repens* roots. Transverse sections of roots were hand-made with razor blades. Fungal cultures were grown on Petri dishes with malt extract agar covered with sterile cellophane film. Mycelium

taken from film was placed on the glass and dyed with 0.01 µg/ml DAPI solution for 1 minute. Slides were examined under Axioscop 40 FL fluorescence microscope (Carl Zeiss, Germany) using DAPI narrow Zeiss filter with 365 nm excitation.

Pathogenicity test

Pathogenicity of Ceratobasidiaceae isolates was studied on potato tuber slices. Healthy potato tubers were washed, surface-sterilized in 0.5% sodium hypochlorite solution for 15 min, rinsed in distilled water, peeled and sliced with a sterile blade. Slices were put into sterile wet chambers. Actively growing mycelium was placed in the center of the slice and incubated at 12^{o}C for 7 days and then at 24^{o}C for other 7 days. Pathogenicity was measured as radiuses of mycelial growth after 7 and 14 days. Four potato pathogenic strains of *Rhizoctonia solani* J.G. Kühn: R156, P1, P2 and K3- 3 isolated from infected potato in Russia were used as positive control. In addition to the strain revealed in the current study, two strains of orchid mycobionts were used in the experiment: Zs5-1 (from *Zeuxine strateumatica* (L.) Schltr.) and Ss1-1 (from *Spiranthes hongkongensis* S.Y. Hu & Barretto) isolated from plant roots in Shenzhen, China. The experiment was conducted with three replicates for each strain.

Electron microscopy

Ultrastructure of Ceratobasidiaceae isolates (same as in “Pathogenicity test”) was studied by scanning electron microscopy (SEM) on equipment of the Center for collective use “Electron microscopy laboratory of Moscow State University Biology Faculty”.

Mycelium was grown on malt agar medium for 7 days and fixed in 2.5% glutaraldehyde. Samples were dehydrated in ethanol, dried in Hitachi HCP-2 critical point dryer (Hitachi, Japan), coated by Eico IB-3 ion coater (Eico, Japan) and examined under JSM-6380 scanning electron microscope (JEOL Inc, USA).

Data analysis

Fungal taxa were identified by ITS sequences using GenBank and UNITE databases, aligned by MAFFT algorithm, and analyzed in MEGA-X. Maximum-likelihood phylogenetic tree was calculated in IQTree online service and visualized in FigTree v. 1.4.4. Scientific names of fungal taxa are given according to MycoBank database (MycoBank 2023).

Results

Biodiversity and occurrence of G. repens mycobionts on studied territories

Fungal sequences (OTUs — operational taxonomic units) revealed by metagenomic approach by themselves could not indicate the certain fungal clone as a mycobiont of *G. repens.* However, introduction of additional requirements regarding known ecological role and occurrence of certain clone in the sample may narrow down the list of putative mycobionts. Therefore, to assume a certain clone as a putative OM mycobiont, it was obliged to fulfill the following requirements: (1) to appear among dominant 25% of OTUs in *G. repens* root sample; (2) do not appear in dominant 25% of OTUs in matching soil sample; (3) to appear among the taxa known as OM symbionts. These requirements were applied to basidiomycete taxa with *Rhizoctonia* anamorphs containing OM mycobionts: Sebacinaceae, Tulasnellaceae and Ceratobasidiaceae.

Sebacinaceae OTUs appeared more frequently in soil samples and lacked specificity towards the roots of *G. repens*, Tulasnellaceae OTUs were not revealed in studied samples. Thus, Ceratobasidiaceae is the only group of orchid mycobionts, members of which could be assumed as putative mycobionts (see supplementary materials, Figure S1). High share of Ceratobasidiaceae in *G. repens* roots and presence of pelotons (see supplementary materials, Figure S2) proves that revealed OTUs are related to mycorrhizal fungi.

Allover, metagenomic analysis revealed 5 Ceratobasidiaceae OTUs: 4 belonging to *Ceratobasidium* and one – to *Thanatephorus.* Due to uncertain phylogeny of Ceratobasidiaceae and lack of highly similar reference sequences, revealed *Ceratobasidium* OTUs were identified to genus level. Species were delimited based on less than 95% match (see Table 2).

Table 2
Revealed orchid mycorrhizal ceratobasidioid taxa. L – Leningrad region, M – Moscow region, T – Karachay-Cherkessia

Taxon	GenBank number	Origin	Putative mycobiont	Isolation method
Ceratobasidium sp1	OP782630.1	M	M,	Culture
	OQ244428.1	L, M, T	M, T	Metagenome
Ceratobasidium sp5	OP800122.1	L, M	L	
Ceratobasidium sp6	OP800123.1	L	L	
Ceratobasidium sp7	OP782636.1	T	T	
Thanatephorus ochraceus	OP782644.1	M	M	

Among revealed OTUs only *Ceratobasidium* sp1 was detected in all regions studied and *Ceratobasidium* sp5 was revealed in Leningrad and Moscow regions. *Ceratobasidium* sp6, 7 and *Thanatephorus ochraceus* (Massee) P. Roberts were specific for Leningrad region, Karachay-Cherkessia, and Moscow region correspondingly.

Isolate OP782630.1 from M3 site was obtained using cultural method. ITS2 region of this isolate matched *Ceratobasidium* sp1 and its mycelium was binucleate.

Shares of Ceratobasidiaceae OTUs in roots of *G. repens* ranged from 12–85% of all fungal OTUs per sample (see Fig. 1). The highest shares (> 70%) were revealed on sites L1, L2, and T4 with no conifer trees nearby.

Allover, 5 Ceratobasidiaceae OTUs were assumed as *G. repens* mycobionts in the studied regions: *Ceratobasidium* sp5 and 6 in Leningrad region, *T. ochraceus* and *Ceratobasidium* sp1 in Moscow region and *Ceratobasidium* sp1 and 7 in Karachay-Cherkessia.

Global distribution of revealed ceratobasidioid mycobionts

Deeper analysis of revealed Ceratobasidiaceae OTUs distribution and ecology was made by investigation of reference sequences from GenBank database that were highly similar (> 97%) to revealed OTUs (see Table 3).

Table 3
References for revealed Ceratobasidiaceae with 97% similarity threshold

Reference ID	Similarity, %	Host	Region
***Ceratobasidium* sp1**			
MH855688.1	99,03	*Quercus pedunculata*	Italy
MH248045.1	99,03	*Goodyera repens*	Moscow region, Russia
AJ419929.1	99,03	*Pinus sylvestris*	Finland
KP056301.1	98,46	*Goodyera repens*	Norway
EU668908.1	98,46	*Pyrola rotundifolia*	Estonia
KF646110.1	97,49	*Rosa rugosa*	Lithuania
JQ972064.1, JQ972069.1, JQ972066–67.1, JQ972072–73.1	97,3	*Platanthera yadonii*	California, USA
GQ268595.1	97,3	Dipterocarpaceae	Malaysia
Ceratobasidium sp5			
KU516417.1	100	*Abies alba*	Poland
OL437012.1	98,88	*Pinus taeda*	Idaho, USA
MK397197.1	97,49	*Pinus greggii*	Mexico
Ceratobasidium sp6			
KP056302.1	99,45	*Goodyera repens*	Norway
MZ078478.1	99,17	*Quercus robur*	Poland
DQ309181.1	98,62	*Calluna vulgaris*	Australia
JQ972107.1, JQ972109.1, JQ972111.1, JQ972113–17.1	98,06	*Platanthera yadonii*	California, USA
MW927755–56.1, MW927758.1, MW927767.1, MW927770–71.1	97,79	*Platanthera cooperi*	USA
Ceratobasidium sp7			
MN006062.1	98,9	*Gymnadenia conopsea*	ND
HM141046.1	98,34	*Goodyera velutina*	Japan
HM141010.1	97,79	*Goodyera tesselata*	Massachusetts, USA
T. ochraceus			
MN684576.1	99,23	*Taeniophyllum glandulosum*	China
AB831841.1	97,69	*Neottia* sp.	Japan
EU218892.1	97,44	Orchidaceae gen sp.	ND
FJ788721–22.1	97,18	*Pterygodium alatum*	South Africa

Ceratobasidium sp1 references were revealed in roots of *G. repens* and related habitats in conifer forests: roots of *P. sylvestris* and *Pyrola rotundifolia.* Also they were detected in association with *Platanthera yadonii* orchid and in unrelated hosts: *Rosa rugosa, Quercus pedunculata* and Dipterocarpaceae. *Ceratobasidium* sp5 references were revealed in association with conifer trees in Poland, USA, and Mexico. *Ceratobasidium* sp6 conspecifics were reported from *G. repens* roots in Norway and conifer forests (*Calluna vulgaris*) in Australia. Its match was also found in association with orchids *P. yadonii* and *P. cooperi* and in deciduous forests (*Quercus robur*). *Ceratobasidium* sp7 conspecific was found associated with two *Goodyera* species: *G. velutina* and *G. tesselata* and orchid *Gymnadenia conopsea. T. ochraceus* references were also found in association with different orchids.

All revealed OTUs except *Ceratobasidium* sp5 were previously reported in orchid roots. Highly similar references of *Ceratobasidium* sp1 and *Ceratobasidium* sp6 were revealed in *G. repens* roots from Moscow region and Norway. *Ceratobasidium* sp5, 7 and *T. ochraceus* are reported in roots of this species for the first time.

Phylogeny of Goodyerinae mycobionts

Host and region specificity of ceratobasidioid mycobionts of Goodyerinae orchids was investigated via ITS2 phylogeny. ITS sequences of Ceratobasidiaceae isolated from Goodyerinae hosts of genera *Anoectochilus, Chamaegastrodia, Cheirostylis, Erythrodes, Goodyera, Hataeria*, and *Zeuxine* were obtained from GenBank database. Clades with > 80% statistic support were regarded as a single highly conservative unit to assume specificity to certain plant host or geographic region. The latter were highlighted on a global scale: Pacific region includes Japan and western USA, Atlantic region includes Norway, western Russia and eastern USA, and tropical region includes Taiwan, Southern China, Thailand, India, Hawaii, and Puerto-Rico.

Outer group was formed by three representatives of other Cantharellales families: Botryobasidiaceae (*Botryobasidium robustius* Pouzar & Hol.-Jech. MH859491.1), Tulasnellaceae (*Tulasnella cumulopuntioides* S. Fujimori, J.P. Abe, I. Okane & Y. Yamaoka NR_160570.1), and Cantharellaceae (*Cantharellus paucifurcatus* Buyck & V. Hofst. NR_137854.1). Also Ceratobasidiaceae representatives of known species isolated from non-orchid hosts were included: *Ceratobasidium angustisporum* Warcup & P.H.B. Talbot NR_154601.1, *C. pseudocornigerum* M.P. Christ. MH861653.1, *C. anceps* (Bres. Syd. & P. Syd.) H.S. Jacks. MH855251.1, *C. papillatum* Warcup & P.H.B. Talbot NR_154600.1, *C. cereale* D.I. Murray & Burpee AJ302008.1, *C. chavesanum* M.P. Melo, J.A. Ventura, H. Costa & P.C. Ceresini NR_164016.1, *C. cornigerum* AJ301900.1, *C. ramicola* C.C. Tu, Roberts & Kimbr. NR_138368.1, *Ceratorhiza oryzae-sativa* (Sawada) R.T. Moore MH861282.1, *Ceratorhiza rhizodes* (Auersw.) Z.H. Xu, T.C. Harr. M.L. Gleason & BatzerMH859145.1, and *Thanatephorus cucumeris* (A.B. Frank) Donk MH855798.1 (see Fig. 2).

A total of 14 highly supported groups were revealed by phylogenetic analysis. Two groups restricted to Europe were also united by plant host *G. repens*. Two groups were specific for Pacific region and include fungi isolated from *Goodyera* spp. and *Hataeria* spp. Five groups specific for tropical region were revealed in *Anoectochilus* spp., *Zeuxine* spp. and *Erythrodes plantaginea.* Five groups were not restricted to the certain region.

Clones *Ceratobasidium* sp1 and 6 were clustered with sequences isolated from *G. repens* (clades II, III), with *Ceratobasidium* sp5 on a sister clade with low support (48%). *Ceratobasidium* sp7 was clustered on a mixed clade IV with fungi isolated from *G. repens, G. tesselata* and *G. schlechtendaliana* from Europe, USA and Japan. *T. ochraceus* was clustered with a mycobiont of *G. procera* from Japan on a mixed clade VIII.

Orchid mycobionts do not cluster with sequences of known Ceratobasidiaceae species so the identification remains on the genus level.

Cultural characteristics and pathogenicity of mycorrhizal and pathogenic isolates

Anatomy of isolated strain *Ceratobasidium* sp1 was studied in comparison with isolates of 4 plant pathogens and 2 orchid mycobionts. Strains R156 and K3-3 with teleomorph *Thanatephorus cucumeris* AG 3 were isolated from potato tubers. Strains P1 and P2 isolated from potato stem had teleomorph *Ceratobasidium* sp. AG K. Two orchid mycobionts Zs5-1 and Ss1-1 were isolated from *Zeuxine strateumatica* and *Spiranthes hongkongensis* and had teleomorph *Ceratobasidium* sp. AG F and L correspondingly.

Nuclear status was revealed using fluorescence microscopy. SEM method was used to observe polysaccharide sheath (see Table 4, Figure S3).

Table 4
Cultural characteristics and pathogenicity of pathogenic and mycorrhizal Ceratobasidiaceae isolates

Pathogenicity, 14 days, 24°C, mm	Pathogenicity, 7 days, 12°C, mm	Polysaccharide sheath	Nuclei	Anastomosis group	Plant host	Origin	Nutrition mode	Taxon	Isolate
6.6 ± 2.0	4.2 ± 1.9	Weak	> 2	AG 3**	Potato, tuber	Moscow region	Plant pathogen	*Thanatephorus cucumeris*	**R156**
30.7 ± 8.5	6.0 ± 1.1			AG 3		Smolensk region			**K3-3**
8.0 ± 1.7	5.0 ± 0.0	Massive	2	AG K	Potato, stem	Astrakhan region		*Ceratobasidium* sp.	**P1**
12.3 ± 2.5	NP*			AG K					**P2**
7.0 ± 0.8	4.3 ± 0.5			ND***	*G. repens* root	Moscow region	Mycorrhizal		**C sp1**
9.25 ± 2.2	NP			AG F	*Zeuxine strateumatica* root	Shenzhen, China			**Zs5-1**
9.25 ± 1.0	NP			AG L	*Spiranthes hongkongensis* root				**Ss1-1**
*NP – pathogenicity not observed									
**Anastomosis groups were identified by closest reference ITS sequence									
**ND – no data									

Polysaccharide sheath surrounding hyphae varied in structure and was measured by “weak” and “massive” categories. Weak sheath of thickness commensurable to cell wall thickness surrounds single hyphae, eventually being observed in branching points and between hyphae for pathogenic multinucleate *Thanathephorus* isolates on SEM (Figure S3). Massive sheath is thicker than cell wall, surrounds multiple hyphae and clearly seen on SEM on a surface of hyphae. This type of sheath is intrinsic for pathogenic and mycorrhizal binucleate *Ceratobasidium* isolates.

Four out of seven studied isolates show pathogenicity on potato tubers under 12^{o}C. Two OM isolates: Zs5-1 and Ss1-1 and pathogenic strain P1 did not show any growth under these conditions. Still, no significant difference between pathogenicity areas of OM and pathogenic isolates was shown ($p > 0.05$). After incubation under 24^{o}C all isolates have shown pathogenicity with no significant difference between OM and pathogenic strains ($p > 0.05$).

Discussion

Biodiversity of ceratobasidioid mycobionts of G. repens

Ceratobasidiaceae are known to be the most abundant of *Goodyera* mycobionts (Shefferson et al. 2015). In the current research we proved the preference of this association and elucidate internal taxonomical and regional specificity of Goodyerinae species and its mycobionts.

Allover, 5 putative mycobionts of Ceratobasidiaceae family were revealed in roots of *G. repens*, 3 of which were revealed in roots of this species for the first time and 2 OTUs were not previously reported in roots of *Goodyera* spp.

Ceratobasidium sp1 was previously revealed in roots of *G. repens* in northern Europe: Norway (Liebel et al. 2015) and Moscow region of Russia (Voronina et al. 2018). This isolate also had high similarity (99%) with *Rhizoctonia quercus* E. Castell. isolated from *Quercus pedunculata* roots in Italy. However, this reference isolate is uninucleate anamorph (Castellani, 1934) and connection between these two stages is doubtful. *Ceratobasidium* sp1 was the only isolate revealed from all three studied regions that allows us to broaden the distribution area of this taxon in association with *G. repens* from Norway and Leningrad region on the north to Caucasus Mountains on the south. This isolate was clustered with mycobionts of *G. repens* which allows us to presume that these isolates belong to one species that is specific for *G. repens* in Europe and USA.

Ceratobasidium sp5 was revealed in association with orchid roots for the first time. However, the presence of highly similar isolates in conifer forests may presume its occurrence in OM in Europe and USA. This OTU was revealed in Leningrad and Moscow region and assumed as a mycobiont in Leningrad region. This isolate did not belong to a branch with high support but was close to clade II represented by mycobionts of *G. repens*.

Ceratobasidium sp6 is a putative mycobiont in Leningrad region and is highly similar to *Ceratobasidium* sp. previously revealed from *G. repens* in Norway. These sequences formed a conservative clade III distant from clade II also specific for *G. repens*. Although these clades were uniform by region and plant host, phylogenetic analysis assumes them as evolutionary diverse.

Ceratobasidium sp7 was specific for Karachay-Cherkessia in 5 of 6 studied sites which assumes its domination in this region with occasional substitution by *Ceratobasidium* sp1. Similar sequences were found in roots of *Goodyera* spp. and *Gymnadenia conopsea* orchids in Japan and USA which assumes wide host and geographic range of this taxon. Domination of this mycobiont in Karachay-Cherkessia and its absence in Moscow and Leningrad regions may be conditioned by subalpine ecosystem with domination of *Abies nordmanniana* and altitudinal zoning: *G. repens* clones were not found below 1675 m. These characteristics discriminate this region and affect mycobiome of *G. repens*.

T. ochraceus was the only clone identified to species level by ITS region. This species is known to form mycorrhiza with wide range of orchid hosts (Roberts 1998). Similar sequences were revealed in association with various orchids in China, Japan and South Africa which assumes wide geographical range of this species as orchid mycobiont.

Therefore, revealed mycobionts may possess different ecological ranges. *Ceratobasidium* sp1, 5 and 6 are presumably specific for conifer forests in northern hemisphere, whereas *Ceratobasidium* sp7 and *T. ochraceus* possess a wide geographic range in association with orchid roots which is also proved by their close relationship with mycobionts of *G. schlechtendaliana* and *G. procera* in Japan. Studying evolutionary specificity of Ceratobasidiaceae is challenging due to uncertain phylogeny and difficulty of ecological preference estimation. Nonetheless, revealing geographic and ecological specificity of ceratiobasidioid isolates would elucidate evolution perspectives for emergence of clades specific for certain orchid taxa.

Specificity of Goodyerinae hosts

Previous extensive study on *Goodyera* mycorrhizal specificity assumed narrow specificity of *G. repens* based on phylogenetic distance between revealed mycobionts (Shefferson et al. 2010). Biodiversity of mycobionts revealed in the current study indicates ability of *G. repens* to form OM with broad range of distantly related mycoiont taxa. Breadth of involved mycobionts is revealed down to regional scale where in each studied region 2 putative ceratobasidioid mycobionts are shared. Re-qualification of *G. repens* to generalist species along with *G. foliosa, G. velutina* and *G. procera* (according to Shefferson et al. 2010) illustrates necessity to provide extensive studies on mycorrhizal specificity of certain orchid species to reveal evolutionary traits and generalize information on a scale of tribes and subfamilies.

Phylogeny of Goodyerinae mycobionts suggests that conservative evolution lines of European and tropical mycobionts are diverse. That illustrates specificity of mycorrhizal Ceratobasidiaceae on a global scale regarding region and plant host. Extensively studied tropical orchid *Anoectochilus formosanus* shows broad mycobiont range with no common clades in Atlantic region. Orchids in Pacific region, by contrast, tend to associate with phylogenetically diverse mycobiont taxa which are present on mixed clades sharing them with either Atlantic or tropical orchids.

Orchid mycobionts and ectomycorrhizal fungi

Various orchid species are known to form mycorrhizal association with ectomycorrhizal fungi. In the current research we spotted a curious tendency that *G. repens* clones distant from conifer trees contain the highest share of Ceratobasidiaceae fungi. Sites L1, L2 and T4 simultaneously possess low shares of ectomycorrhizal fungi from Agaricales, Russulales and Atheliales, while sites M2 and T3 with lowest Ceratobasidiaceae share possess the highest share of ectomycorrhizal fungi (see supplementary materials, Figure S4). These data assume a competition between these two symbiotic groups in orchid roots and a potential for mycorrhizal networks formation between *G. repens* and surrounding conifers. However, a more extensive study is needed to elucidate an interaction between orchid mycobionts and ectomycorrhizal fungi.

Cultural characteristics and pathogenicity

In the current study we made an attempt to establish possible connection between ceratobasidioid isolates nutritional modes and morphological characteristics. Polysaccharide sheath was measured due to ability to prevent decomposition by plant β-glucanases. Significant difference between mycorrhizal and pathogenic strains has not been revealved, but sheath thickness differs between *Ceratobasidium* and *Thanatephorus* isolates. Variability of these two parameters may indicate different adaptations of Ceratobasidiaceae to plant-associated life style. Massive sheath might be beneficial for enduring existence inside living plant cells that is typical for OM symbionts, whereas weak sheath may be a trait of necrotrophic pathogens that aim on quick decomposition of plant cells. However, large variety of Ceratobasidiaceae nutritional modes from pathogens to mutualists assumes existence of transition states of these characteristics.

Pathogenicity of Ceratobasidiaceae isolates was studied towards potato tubers. Ability of both mycorrhizal and pathogenic isolates to decompose tubers under 24^{o}C may witness that even OM strains may show pathogenic activity under certain conditions. Moreover, under favorable conditions (24^{o}C) no significant differences between OM and pathogenic isolates were found. Inability of three strains to grow on potato tuber under 12°C may be explained by their distribution area: tropical China and Astrakhan region of Russia.

Conclusion

Studies of biodiversity and phylogenetic relations of ceratobasidioid mycobionts shed light on OM evolution and specificity. It the current study with the example of *Goodyera repens* we prove that orchids may be associated with a wide range of distantly related Ceratobasidiaceae embracing both generalist and narrowly specialized taxa. This diversity is shown on regional and global scales illustrating generalism of *G. repens*.

Cultural isolation of mycobionts with further identification should be supplied with at least indirect evidence towards their ecological role as far as it remains the most accurate way to identify the certain isolates which form mycorrhizas with orchids.

Competition between orchid mycobionts and ectomycorrhizal fungi is still to be studied in context of preferences given by orchid and potential for assumable mycorrhizal network establishment. Our data assume that *G. repens* plants located in the area that is accessible for conifer roots may partly substitute ceratobasidioid mycobionts with ectomycorrhizal fungi. This fact points at possibility of mycorrhizal formation between *G. repens* and ectomycorrhizal fungi and assumes ectomycorrhizal plant roots accessibility as an important factor that shapes community of orchid mycobionts. Further studies on anatomy and physiology of this association are required to understand this peculiar interaction.

Morphological characteristics may partly reflect the nutritional mode of Ceratobasidiaceae isolates, but wide range of trophic states might generate a variety of transitional stages with ambiguous delimitation.

Ability of mycorrhizal strains to decompose potato tubers with rates comparable to those of pathogenic isolates assumes ability of OM Ceratobasidiaceae to show pathogenicity under certain conditions.

Declarations

Author contribution

NB, EV and AK designed experiment, NB and AK performed sample collection, NB performed culture isolation and metagenome data analysis, EV wrote the main part of the manuscript, OK performed electron microscopy, MY performed pathogenicity tests. All authors reviewed manuscript.

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Competing interests.

The authors declare no competing interests.

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Figures

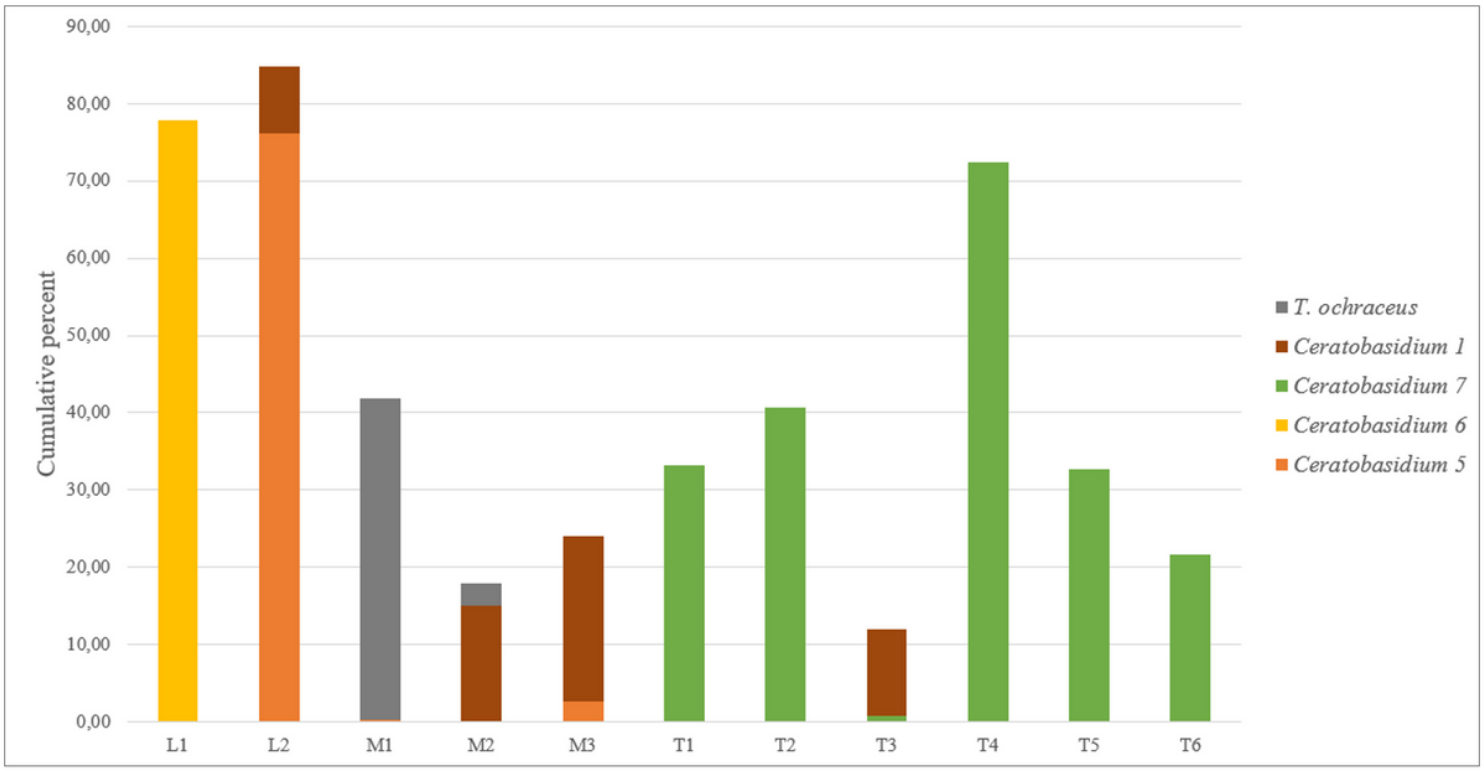

Figure 1

Shares of Ceratobasidiaceae members OTUs in *G. repens* root samples

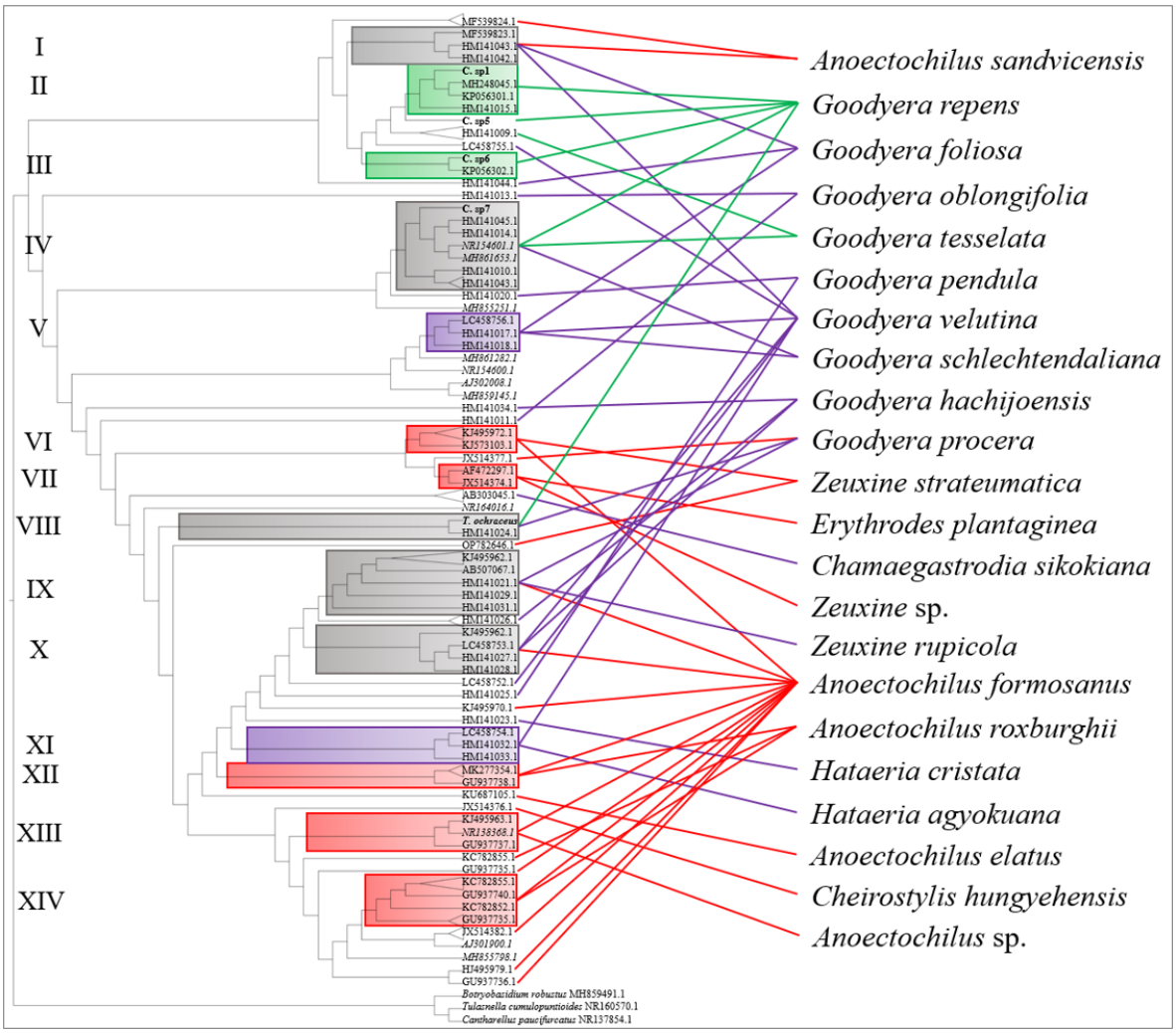

Figure 2

ITS2 Maximum likelihood tree of *Ceratobasidiaceae* fungi connected to their Goodyerinae hosts. Identical sequences from similar hosts and habitats are united and displayed as triangles. Purple – Pacific region; green – Atlantic region; red – tropical region; grey – mixed group. Numbers of sequences obtained in current research are marked in bold. Non-OM sequences are marked in italic.

Supplementary Files

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