Fungi in Living and Dead Stems and Stumps of *Pinus mugo* on Coastal Dunes of the Baltic Sea

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Abstract

Lygis V., Vasiliauskaite I., Matelis A., Pliūra A., Vasaitis R. (2014): **Fungi in living and dead stems and stumps of *Pinus mugo* on coastal dunes of the Baltic Sea**. Plant Protect. Sci., **50**: 221–226.

Communities of xylotrophic fungi were studied in wood of *Pinus mugo* of different qualities: (*i*) living stems, (*ii*) cut stumps, (*iii*) burned snags, (*iv*) cut burned stumps, (*v*) stems recently killed by root rot, and (*vi*) old snags of root rot-killed trees. A total of 277 isolates representing 58 fungal taxa were obtained from 300 wood samples (50 samples per each substrate category). Results of the present study suggested that following different disturbances (tree felling, forest fire or root rot), fungal communities likely evolve in different directions: depending on its origin (cut, burned or killed by the disease), dead wood might be inhabited by principally different microbial assemblages, and that fire has less effect on community structures than tree felling or root rot.

Keywords: forest fire; fungal diversity; mountain pine; mycobiota; root rot; succession

Mountain pine (*Pinus mugo* Turra) is an alien pine species introduced to Lithuania in the beginning of the 19th century in order to stabilise moving sand of the coastal dunes in Curonian Spit peninsula located on the south-eastern coast of the Baltic Sea. Nowadays *P. mugo* comprises a unique ecosystem component of the Kuršių Nerija National Park, which is included in the list of the UNESCO World Heritage Sites. Currently, the area of *P. mugo* plantations in the Curonian Spit is decreasing due to: (*i*) clear-cuts to be replanted by native *P. sylvestris* (L.), (*ii*) regularly occurring forest fires (the peninsula is a popular recreational site), and (*iii*) mortality caused by root rot fungi *Heterobasidion* spp. and *Rhizina undulata* L. (the latter is fire-associated pathogen) (Vasiliauskas 1999; Lygis *et al.* 2010).

As a consequence, *P. mugo* ecosystems in the Curonian Spit presently include areas of several distinctly different site types: (*i*) apparently vigorous forest stands, (*ii*) their deliberate (planned) clear-cuts, (*iii*) burned standing forest, (*iv*) sanitary clear-cuts of burned forest, (*v*) recent, and (*vi*) old root-rot disease centres. As evident, those sites feature woody substrates of different qualities, respectively: (*i*) living stems, (*ii*) cut stumps, (*iii*) burned snags, (*iv*) cut burned stumps, and root-rot disease centres comprised of (*v*) recently, and (*vi*) long ago root rot-killed trees (old snags). While fungi that inhabit living trees have a potential impact on tree health condition, the fungi colonising dead wood (e.g. stumps and snags, or coarse woody debris (CWD)) are the major drivers behind decomposition, mineralisation, and turnover

Presented at the IUFRO Meeting "Biosecurity in Naturals Forests, Stands and Plantations, Genomics and Biotechnology for Biosecurity in Forestry", Brno and Cerna Hora, Czech Republic, May 20–25, 2013.

of organic matter, which is of crucial importance in forest ecosystems characterised by poor soils (e.g. sandy dunes). Clear-felling operations initially favour stump colonisation by fungi with primary resource capture strategies such as the economically important pathogen *Heterobasidion annosum* (Fr.) Bref. *sensu lato* which subsequently causes mortality and/or root and butt rot of different tree species on infested sites (Woodward *et al*. 1998; Garbelotto & Gonthier 2013, and references therein). In Lithuania, pine stands (especially pure plantations of *P. sylvestris* and *P. mugo*) are commonly devastated by *H. annosum sensu stricto* often resulting in open areas (infection foci) of up to 0.5 ha which are non-productive and may comprise thousands of hectares on a country-wide scale (Vasiliauskas 1989, 1999; Lygis *et al*. 2004a). It is known that communities of wood-inhabiting fungi change along with changes in tree condition (Lygis *et al.* 2004a,b, 2005; Giordano *et al.* 2009); therefore, by having a great impact on condition of the infected tree, annosus root rot disease could be regarded as one of the major factors affecting composition of wood mycobiota in pines.

To date, only two published studies have been available on mycobiota in CWD of *P. mugo*, and in fact, both these studies were carried out in the Curonian Spit. Thus, Lygis *et al.* (2010) investigated fungal communities in coarse decayed sub-soil roots in burned and non-burned sites, and found out moderately similar fungal communities on both site types, but also suggested that forest fires on sandy soils have a certain potential to reduce the occurrence of *Heterobasidion* root rot. Kutorga *et al.* (2012a,b) recorded fungal fruiting structures on a wide variety of organic substrates (herbs, litter, leaves, mosses etc., including non-specified "woody material") and observed significantly different species compositions in "burned unmanaged", as compared with "burned clear-cut" *P. mugo* sites. So far, published studies on endophytes inhabiting living *P. mugo* stems have been absent.

Results from our previous studies (Lygis *et al.* 2004a,b, 2005) clearly demonstrated that: (*i*) actual fungal diversity in snags, stumps, and living stems drastically exceeds the diversity which is externally manifested on the substrates by sporocarp formation; (*ii*) prevailing part in those communities consists of micromycetes, fruiting structures of which are simply not detectable under field conditions; (*iii*) a large proportion of those fungi could be identified only using molecular methods (as they form any distinctive morphological structures neither *in vivo*, nor on nutrient media); and (*iv*) considerable part of the community will nevertheless remain unidentified, but is of value for possible comparative studies. Therefore, the aim of the present work was to provide a molecular characterisation of fungal communities in living stems, and in CWD of *P. mugo* on site types subjected to clear-cuts or fire, or both those types of disturbance.

MATERIAL AND METHODS

Study area. The study area is represented by 120-year-old *P. mugo* plantations established on sandy dunes in the Curonian Spit (55°39'N, 21°07'E), and typically included *Heterobasidion* disease centres. In May 2006, a severe forest fire devastated approximately 250 ha of the stands in the area (Lygis *et al.* 2010), and the most of burned area was clear-felled in July 2006, while in some parts groups of the burned snags were retained. At the same time, a path of living (vigorous) pines was cut around the perimeter of the burned area.

Sampling, isolation, and identification of fungi. In August 2008 (27 months after the forest fire and 24 months after the felling), the following categories of woody substrates were sampled in the study area: (*i*) living (vigorous) stems, (*ii*) cut stumps of living trees, (*iii*) burned snags (stems charred up to the top, crowns scorched completely), (*iv*) cut stumps of burned snags, (*v*) stems of trees recently killed by the root rot (during last year: brown attached needles present), (*vi*) old snags of trees killed by the root rot (at least 5 years ago: no twigs/needles, but thick, lichen-covered branches and stems). Fifty stems or stumps were sampled in each category. The mean diameter of the sampled stems (trees and snags) was about 5 cm at breast height, the mean height was about 4 m. The mean stump diameter was 7.0 cm ranging between 5 and 10 cm. The distance between two closest sampling units in each plot ranged 0.5–1.0 m. Sampling of wood for fungal isolations was performed as described by Lygis *et al.* (2004a). Briefly, two bore cores (4 × 50 mm) were extracted with an increment borer from the opposite sides of a root collar (5 cm above ground) of each stem or stump. The sampled wood cores were placed into separate sterile hermetically closed plastic tubes and transported to the laboratory.

In the laboratory, isolation and identification of fungi was made as in our previous studies (Lygis *et al.* 2004a,b, 2005). First, the isolates were examined under a light microscope and distributed into groups based on mycelial morphology (grouped together

Table 1. Frequencies (%) of isolation of fungal taxa from living stems, snags, and stumps of *Pinus mugo*

Fungal taxa	GenBank accession-number	Substrate types[a]						Total
		living stems	sound stumps[b]	burned snags	burned stumps[c]	recently dead stems[d]	old snags[e]	
Basidiomycetes								
Agaricomycetes sp. VL164	JF440567	–	–	–	2.0	–	–	0.3
Agaricomycetes sp. VL291B	JF440568	–	–	2.0	–	–	–	0.3
Coniophora arida (Fr.) P. Karst.	JF440569	–	–	–	–	–	4.0	0.7
Coniophora puteana (Schumach.) P. Karst.	JF440570	–	–	2.0	2.0	–	–	0.7
Fomitopsis pinicola (Sw.) P. Karst.	JF440571	–	–	14.0	–	–	–	2.3
Heterobasidion annosum (Fr.) Bref.	JF440572	–	–	–	–	2.0	–	0.3
Peniophora cinerea (Pers.) Cooke	JF440573	–	–	2.0	–	–	–	0.3
Phanerochaete sordida (P. Karst.) J. Erikss. & Ryvard.	JF440574	–	–	–	2.0	–	–	0.3
Phlebia radiata Fr.	JF440575	–	–	–	2.0	–	–	0.3
Phlebia sp. VL297	JF440576	–	–	–	–	2.0	–	0.3
Phlebiopsis gigantea (Fr.) Jülich	JF440577	6.0	–	10.0	–	–	–	2.7
Pholiota highlandensis (Peck) Quadr. & Lunghini	JF440578	2.0	–	2.0	–	–	–	0.7
Schizophyllum commune Fr.	JF440579	–	2.0	–	2.0	–	–	0.7
Stereum sanguinolentum (Alb. & Schwein.) Fr.	JF440580	–	2.0	–	–	–		0.3
All basidiomycetes		8.0	4.0	30.0	10.0	4.0	4.0	10.0
Ascomycetes & anamorphic fungi								
Alternaria alternata (Fr.) Keissl.	JF440581	–	12.0	–	14.0	–	–	4.3
Apiospora montagnei Sacc.	JF440582	–	2.0	–	4.0	–	–	1.0
Ascocoryne cylichnium (Tul.) Korf	JF440583	–	–	4.0	2.0	–	16.0	3.7
Aureobasidium pullulans (de Bary) G. Arnaud.	JF440584	–	–	–	–	–	2.0	0.3
Ceratocystis minor (Hedgc.) J. Hunt	JF440585	–	–	–	–	2.0	–	0.3
Chromelosporium carneum (Pers.) Hennebert	JF440586	2.0	–	–	2.0	–	–	0.7
Cladosporium tenuissimum Cooke	JF440587	2.0	–	–	–	–	–	0.3
Cytospora sp. VL189	JF440588	–	–	–	4.0	–	4.0	1.3
Cytospora sp. VL218	JF440589	–	6.0	2.0	18.0	–	–	4.3
Epicoccum nigrum Link	JF440590	–	4.0	–	24.0	–	–	4.7
Exophiala sp. VL279	JF440591	2.0	–	–	–	–	–	0.3
Fimetariella rabenhorstii (Niessl) N. Lundq.	JF440592	4.0	–	2.0	–	–	–	1.0
Fusarium oxysporum Schltdl.	JF440593	–	–	–	2.0	–	–	0.3
Fusarium sporotrichioides Sherb.	JF440594	–	–	2.0	–	–	–	0.3
Gelasinospora sp. VL222	JF440595	–	2.0	–	6.0	–	–	1.3
Gibberella avenacea Cook	JF440596	–	–	–	4.0	–	–	0.7
Lachnellula sp. VL403	JF440597	–	–	–	–	–	2.0	0.3
Lecythophora hoffmannii (Beyma) W. Gams & McGinnis	JF440598	8.0	–	16.0	–	–	–	4.3
Leptodontidium elatius (F. Mangenot) de Hoog	JF440599	–	–	–	–	–	2.0	0.3
Leptographium procerum (W.B. Kendrick) M.J. Wingfield	JF440600	–	2.0	–	–	–	–	0.3
Lewia infectoria (Fuckel) M.E. Barr & E.G. Simmons	JF440601	–	2.0	–	–	–	–	0.3
Penicillium chrysogenum Thom	JF440603	4.0	6.0	6.0	10.0	–	16.0	7.0
Penicillium citreonigrum Dierckx	JF440604	–	–	–	–	–	2.0	0.3
Penicillium miczynskii K.M. Zalessky	JF440605	–	–	–	–	–	4.0	0.7
Pezicula sp. VL210	JF440606	–	2.0	–	–	–	–	0.3
Phaeomoniella effusa Damm & Crous	JF440607	–	–	–	–	–	4.0	0.7
Phialophora lagerbergii (Melin & Nannf.) Conant	JF440608	–	–	2.0	–	–	–	0.3
Phoma herbarum Westend.	JF440609	–	–	–	2.0	–	–	0.3
Pleonectria cucurbitula (Tode: Fr.) Hir., Rossm. & Chav.	JF440602	–	8.0	–	8.0	–	–	2.7
Pleurophoma cava (Schulzer) Boerema, Loer. & Hamers	JF440610	–	–	–	4.0	–	–	0.7
Rhinocladiella sp. VL270	JF440611	–	–	2.0	–	–	–	0.3
Rhinocladiella sp. VL271	JF440612	–	24.0	2.0	26.0		4.0	9.3

Table 1 to be continued

Fungal taxa	GenBank accession-number	Substrate types[a]						Total
		living stems	sound stumps[b]	burned snags	burned stumps[c]	recently dead stems[d]	old snags[e]	
Sarea difformis (Fr.) Fr.	JF440614	2.0	–	6.0	–	2.0	–	1.7
Sarea resinae (Fr.) Kuntze	JF440615	2.0	–	–	–	–	2.0	0.7
Scleroconidioma sphagnicola Tsuneda, Currah & Thorm.	JF440616	–	–	–	–	–	2.0	0.3
Sphaeropsis sapinea (Fr.) Dyko & B. Sutton	JF440618	–	4.0	–	–	–	–	0.7
Sydowia polyspora (Bref. & Tavel) E. Müll.	JF440619	–	14.0	2.0	30.0	–	–	7.7
Trichoderma viride Pers.	JF440620	–	14.0	12.0	20.0	4.0	32.0	13.7
Trichophaea hybrida (Sowerby) T. Schumach.	JF440621	2.0	–	–	–	–	–	0.3
Ulocladium sp. VL204	JF440622	–	–	–	2.0	–	–	0.3
Xylariaceae sp. VL208	JF440623	–	–	–	2.0	–	–	0.3
All ascomycetes & anamorphic fungi		24.0	46.0	50.0	76.0	8.0	68.0	45.3
Zygomycetes								
Mucor racemosus Bull.	JF440624	0.0	0.0	0.0	0.0	0.0	6.0	1.0
Umbelopsis isabellina (Oudem.) W. Gams	JF440625	0.0	0.0	0.0	0.0	0.0	8.0	1.3
All zygomycetes		0.0	0.0	0.0	0.0	0.0	10.0	1.7
All species		30.0	48.0	72.0	78.0	12.0	70.0	51.7
No. of taxa per sampled tree/stump		0.22	0.34	0.36	0.52	0.10	0.32	0.19

[a]50 of each sampled, 300 substrates in total; [b]stumps of cut living trees without decay symptoms; [c]stumps of cut burned snags; [d]trees killed by the root-rot during last year; [d]snags originating from trees killed by the root rot at least five years ago

were only the isolates with distinctive asexual reproduction structures such as conidiophores, sporangia, oidiophores, and/or separate spores – conidia, sporangiospores, oidia). Representatives from each group were selected for DNA extraction, and were grown for two weeks on liquid Hagem media in static cultures at a room temperature. The extraction of DNA, PCR amplifications, and DNA sequencing followed the study of Kåren *et al.* (1997), and the ribosomal ITS region was sequenced by Macrogen Inc. (Seoul, Korea) using two primers (ITS1F and ITS4). All sequences were checked against available databases: the NCBI BLAST database and the database of the Swedish University of Agricultural Sciences (Uppsala, Sweden). The ITS sequence homology for delimiting fungal taxon (presumed species) was set at 98–100%, and for delimiting at genus level it was 94–97%. All sequences were deposited in GenBank under the accession numbers provided in Table 1. Similarity among fungal communities colonising stems and CWD of various types was compared by calculating Sorensen similarity indices (C_S – qualitative, C_N – quantitative) (Magurran 1988).

RESULTS AND DISCUSSION

A total of 277 isolates representing 58 fungal taxa have been obtained from 300 wood samples. Of those taxa, 44 (75.8%) were assigned to species, 11 (19.0%) identified on the genus level, and 3 (5.2%) have remained unidentified (Table 1). Yet, the frequencies of isolation were low, and almost a half (48.3%) of the samples did not yield fungal growth (Table 1), perhaps due to a very high resin content in *P. mugo* wood, providing generally unsuitable conditions for fungal colonisation. Apparently, this results in low decomposition rates of *P. mugo* CWD. The most similar fungal communities were found in non-burned vs. burned stumps, and in living stems vs. burned snags, where the level of the similarity, according both to qualitative and quantitative Sorensen similarity indices, was moderate (Table 2). This indicates that the impact of fire on mycobiota in those types of substrates is not very significant. By contrast, comparisons of fungal communities between other substrate types revealed low or very low similarities (Table 2), demonstrating that management practices in this respect are likely to have higher impacts than wildfire.

In living stems and burned snags the most regularly isolated fungus was *Lecythophora hoffmannii* (Beyma) W. Gams & McGinnis, while both in unburned and burned stumps the unidentified species from the genus *Rhinocladiella* (sp. VL271) was the most common, and in stems killed by the root rot (both recently and in old snags) the most common species was *Trichoderma viride* Pers. (Table 1). Yet none of the three above-listed fungi were found to be present in all investigated substrate types. Notably, among the

Table 2. Sorensen similarity indices between fungal communities detected in woody substrates of *Pinus mugo*. Qualitative (C_S) on the left side from the diagonal, and quantitative (C_N) on the right side

Substrate	Living stems	Sound stumps[a]	Burned snags	Burned stumps[b]	Recently dead stems[c]	Old snags[d]
Living stems	–	0.06	0.38	0.07	0.08	0.08
Sound stumps[a]	0.07	–	0.24	0.63	0.07	0.22
Burned snags	0.41	0.29	–	0.21	0.12	0.24
Burned stumps[b]	0.16	0.56	0.36	–	0.04	0.26
Recently dead stems[c]	0.13	0.09	0.17	0.06	–	0.07
Old snags[d]	0.15	0.18	0.24	0.24	0.10	–

[a]stumps of cut living trees without decay symptoms; [b]stumps of cut burned snags; [c]trees killed by the root-rot during last year; [d]snags originating from trees killed by the root rot at least five years ago

whole community of ascomycetes (including anamorphic fungi) and zygomycetes detected in the present study (Table 1: 44 taxa), only six species (*Alternaria alternata* (Fr.) Keissl., *Ascocoryne cylichnium* (Tul.) Korf, *Chromelosporium carneum* (Pers.) Hennebert, *Fimetariella rabenhorstii* (Niessl) N. Lundq., *T. viride* and *Umbelopsis isabellina* (Oudem.) W. Gams) have been previously observed on wood of *P. mugo* (Lygis *et al.* 2010; Kutorga *et al.* 2012b). Consequently, 38 taxa of microfungi isolated by us (or 86.4%) in this respect represent new findings, significantly complementing data from the previous work.

By contrast, the majority of isolated macromycetes (Table 1) are known for *P. mugo* from previous studies (Lygis *et al.* 2010; Kutorga *et al.* 2012b). Interestingly, two of the species, *Phlebiopsis gigantea* (Fr.) Jülich and *Pholiota highlandensis* (Peck) Quadr. & Lunghini, were isolated from vigorous symptomless intact stems. Characteristically, more recent use of molecular techniques has revealed latent presence of (increasing) number of wood-decomposing basidiomycetes in visually healthy stems of many tree species, raising questions on infection biology of those fungi (Vasaitis 2013, and references therein).

Finally, we compared fungal communities detected in *P. mugo* (Table 1) with the communities in living stems (sound xylem) and root rot-killed snags of *Pinus sylvestris* L. (Lygis *et al.* 2004a,b; Giordano *et al.* 2009). Frequency of isolation and species richness were about the same, but mycobiota were largely dissimilar, having two species (*Sarea difformis* (Fr.) Fr. and *S. resinae* (Fr.) Kuntze) as the only common taxa in living stems, and another three species (*H. annosum* s.s., *A. cylichnium,* and *U. isabellina*) in snags. We conclude that in most cases different fungal communities are observed in different woody substrates of pine, but that fire has less effect on community structures than tree felling or root rot.

Acknowledgements. Supported by the European Commission Marie Curie Actions (Project Management of coastal forests of Lithuania: sustaining and enhancing forest health through silviculture – LITCOAST), Grant No. MTKI-CT-2006-042622, and by the Swedish Energy Agency (Energimyndigheten).

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Received for publication March 7, 2014
Accepted after corrections May 21, 2014

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