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Short Communication

No support for occurrence of free-living *Cladonia* mycobionts in dead wood

Veera TUOVINEN[a,*], *Måns SVENSSON*[a], *Ariana KUBARTOVÁ*[b], *Elisabet OTTOSSON*[b], *Jan STENLID*[b], *Göran THOR*[a], *Anders DAHLBERG*[b]

[a]*Department of Ecology, Swedish University of Agricultural Sciences, P.O. Box 7044, 750 07 Uppsala, Sweden*
[b]*Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, P.O. Box 7026, 750 07 Uppsala, Sweden*

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ABSTRACT

Lichenised fungi are traditionally assumed to form obligate symbioses with algae or cyanobacteria and to be confined to the surface of their growing substratum. However, in a recent 454 pyrosequencing study of fungal communities in *Picea abies* logs, lichen-forming fungi were detected at a depth of more than 6 cm in dead wood, implying the existence of free-living lichen mycobionts. To determine whether this was the case, we investigated whether *Cladonia* spp., the most frequently encountered mycobionts, occurred in wood without their photobionts. We detected green algae in all samples with records of *Cladonia* spp. Hence, we found no evidence for free-living *Cladonia* mycobionts in wood. We suggest that the detected *Cladonia* DNA in these logs originates from vegetative propagules or thallus fragments dispersed into the logs by animals or water. However, the occurrence of free-living stages of other lichen-forming fungal taxa in dead wood cannot be excluded.

Introduction

Since Schwendener proposed the dual nature of lichens in 1869, the perception of lichens has gradually changed and they are now considered to be minute ecosystems. The lichen symbiosis includes not only the photobiont(s) and the mycobiont, but also various additional fungi and bacteria are associated with the thallus (Lawrey and Diederich, 2003; Grube et al., 2012). Free-living cyanobacteria and some green algal photobionts occur in nature, although their biological significance and commonness of the same strains occurring in both their symbiotic and free-living forms are not clear (Nash, 2008). Given that the majority of lichen-forming fungi can be cultured separately from their photobionts, these fungi are viewed as facultative biotrophs (Honegger, 1998). However, owing to the slow growth rates of these fungi and their inability to reproduce sexually in culture in the absence of a photobiont, they are generally not considered to have free-living life stages in nature (Nash, 2008).

Wedin et al. (2004) reported the first findings of saprotrophy and lichenisation for the same fungal species on different substrata, a phenomenon they called optional lichenisation. These findings suggest that some lichen-

* *Corresponding author*. Tel.: +46 18 672267; fax: +46 18 672890.
E-mail address: veera.tuovinen@slu.se (V. Tuovinen).
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forming fungi can show ecological plasticity and that for part of their lifecycle, or under certain conditions, they may be free-living saprotrophs. The existence of partial saprotrophy in lichens has also been suggested because many lichens have the capacity to produce extracellular enzymes that could be used to decompose organic matter (reviewed in Beckett et al., 2013). However, it is not known whether these enzymes are used to acquire energy from wood. Some lichenicolous fungi are known to exhibit a range of lifestyles, first parasitising and killing the host lichen mycobiont, capturing their photobiont and then establishing a new lichen thallus (e.g., Friedl, 1987). Similarly certain non-lichenised fungi can act as either mutualistic symbionts, saprotrophs or parasites depending on the environmental conditions (e.g., Vasiliauskas et al., 2007; Martos et al., 2009).

Lichens have traditionally been considered to be restricted to the surface of their substratum. However, studies of the lichen–rock interface of endolithic lichens have shown that lichen hyphae are able to penetrate up to 12 mm into the space between mineral particles, constituting a multiple hyphal volume compared with the symbiotic thallus (Bjelland and Ekman, 2005). Furthermore, hyphae of some epiphytic lichens have been reported to penetrate bark and wood, and to have reached the xylem vessels of their host trees (reviewed in Favero-Longo and Piervittori, 2010). However, the identity of hyphae in bark and wood has not been verified by molecular methods.

Next-generation sequencing technologies have increased our ability to detect and to investigate the patterns and processes of fungal communities in their natural environments. When studying fungal communities in dead wood using 454 pyrosequencing, Kubartová et al. (2012) detected several lichen-forming fungal taxa with a low level of DNA abundance at a depth of more than 6 cm in Norway spruce (*Picea abies*) logs, providing the first molecular evidence of lichen inside wood. These results could indicate the presence of lichen mycobionts in a free-living, partly saprotrophic state or that the hyphae of epiphytic thalli had penetrated several cm into the wood.

We tested whether *Cladonia* spp., the most frequently encountered lichen mycobionts found in the wood samples of Kubartová et al. (2012), were present in the wood without their photobionts. We hypothesised that if the detected fungal DNA belonged to free-living mycobionts or deep-penetrating hyphae, algal DNA would be absent from the wood samples.

Materials and methods

DNA-extractions from the wood samples collected by Kubartová et al. (2012) were re-examined. These extractions comprised the total DNA from 353 drilled wood samples from 38 Norway spruce (*P. abies*) logs in two nature reserves in boreal Sweden. The wood samples had been extracted separately from the outer 6 cm and the following inner 6 cm of the wood. The internal transcribed spacer 2 (ITS2) region was used as a barcoding marker for the identification of fungi. In total, 62 operational taxonomic units (OTUs) of potential lichen mycobionts, accounting for 3 % of the total number of sequence reads, were reported by Kubartová et al. (2012). The most frequently found mycobionts that could be reliably identified belonged to the genus *Cladonia*. The most frequent *Cladonia* OTU was recorded in 75 samples (21 %). Identifying *Cladonia* mycobionts to species level was not possible owing to the absence of a barcoding cap in the ITS marker in the genus (Kelly et al., 2011). For a more detailed description of study sites, sampling methods and laboratory procedures, see Kubartová et al. (2012). All samples where *Cladonia* sequences had been initially detected and that had remaining DNA extractions (62) were reanalysed to determine whether algal symbionts were also present.

Given that *Cladonia* photobionts are known to belong to *Asterochloris*, the photobiont search was limited to that genus using the forward primer nrSSU-1780-5′A (Piercey-Normore and DePriest, 2001) in combination with a newly designed algal-specific reverse primer ITSAC2 (5′-CAGACGCTGAGGCr-GACA-3′). ITSAC2 is located in the 3′ end of the 5.8S gene and primarily amplifies *Asterochloris* and some *Trebouxia*. PCR was run for 35 cycles with annealing at 61 °C. Almost all reactions resulted in the formation of multiple bands in the agarose gel. A set of samples with multiple bands was chosen for cloning. Samples with a single band and the PCR products of the cloned samples were Sanger sequenced so that all the different sized bands were sequenced at least once. Sequences were compared against the NCBI database using a standard nucleotide BLAST search. Given that the purpose of the study was to determine the presence or absence of algae in the samples rather than to identify the species more accurately, the identities of the remaining, non-sequenced bands were interpreted from the agarose gel.

Results and discussion

Algal DNA was detected in all 62 samples tested, originating from both outer and inner wood (Table 1). In 53 cases, the algae were assigned to *Asterochloris*. Other common lichen symbiotic algae from the genus *Trebouxia*, in addition to unidentified green algae, were also frequently found (Table 1).

The detection of green algae at a depth of >6 cm in wood was unexpected and did not support our hypothesis of free-living or deep-penetrating *Cladonia* hyphae. Great care was taken to prevent contamination during field sampling (Kubartová et al., 2012); however, the possibility of contamination during field sampling cannot be excluded. The study of Kubartová et al. (2012) was designed to study non-

Table 1 – The number of samples where green algae were detected. The studied samples were the 62 of the 75 wood samples from Norway spruce logs where *Cladonia* were detected in Kubartova et al. (2012) and could be reanalysed in this study. Algal DNA was detected in all samples

Position in the log	Number of samples with		Total number of samples
	Asterochloris sp.	Other green algae	
Outer wood	26	27	33
Inner wood	27	25	29

lichenised fungal communities and, hence, lichens on the logs were not recorded. Consequently, it was not possible to determine whether the detected mycobiont DNA originated from the studied log surfaces. However, several *Cladonia* species were observed in photographs of the logs taken at the sampling time.

Small cracks and fissures can be abundant in logs. We suggest that vegetative propagules or thallus fragments, potentially also free-living photobionts, may be dispersed into logs by wood-dwelling animals such as mites, or by rain or wind. Small but sufficient amounts of light may penetrate through cracks in the decaying wood, allowing algae to photosynthesise and, hence, the detected photobiont DNA could originate from dead, active, or vital but dormant, vegetative propagules or thallus fragments. It is also possible that the detected mycobionts and photobionts were free-living and coexisting but not associating in wood, and/or that free-living photobionts co-occur with deep-penetrating hyphae of epiphytic lichens. The occurrence of *Trebouxia* and other green algae could also be explained by the presence of several other lichenised fungi in the same samples.

Similar to mycorrhizal fungi, the lichen symbiosis has evolved repeatedly in several fungal lineages and from saprotrophic ancestors (Hibbett et al., 2000; Schoch et al., 2009). Both fungal life forms are largely assumed to be obligate biotrophs in nature. However, they can be grown *in vitro* without their plant partners, which suggests that an independent saprotrophic phase in their life cycle might be possible (Talbot et al., 2008; Beckett et al., 2013). We suggest that if the spores of sexually dispersing lichen mycobionts were able to establish and grow as free-living saprotrophs until appropriate photobionts and favourable conditions for the establishment of a lichen thallus were met, this strategy would have a significant fitness value for the mycobiont. The degree of symbiosis in lichens varies between taxa, ranging from parasitic and mutualistic associations to loose contacts between symbionts. Correspondingly, it is possible that the capability and the tendency for saprotrophy varies between mycobiont species. We only screened samples where *Cladonia* mycobionts were present, but several additional mycobionts were reported by Kubartová et al. (2012), including common crustose, foliose and fruticose epiphytes on the bark or wood of Norway spruce. The discovery of various mycobiont taxa in dead wood merits further investigation to determine whether some of these mycobionts have the potential for a free-living saprotrophic phase.

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