Characterization of ectomycorrhizal communities of *Asteropeia mcphersonii* seedlings spontaneously growing in natural forest and in open disturbed areas

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**ABSTRACT**
Forest ecosystem disturbances generate changes including soil changes, generally with loss of topsoil and loss of much of the diversity of microorganisms, which are usually replaced by others that are more adapted to the new conditions. We describe the effect of rainforest clear-cutting in Madagascar, with stripping of surface soil horizons among ectomycorrhizal fungal communities associated with natural *Asteropeia mcphersonii* seedlings. An experimental device was placed on four sites, each including an undisturbed plot with litter and a plot in clear-cut areas on a soil composed of a homogeneous mineral substrate. On each of these eight plots the ectomycorrhizal infection rate was determined and root tips were collected to sequence the internal transcribed spacer. 219 sequences grouped into 59 operational taxonomic units (OTUs) were obtained. The main taxonomic groups were Russulaceae, Boletales and Thelephoraceae. Marked spatial aggregation was observed for OTUs. The disturbance significantly affected the ectomycorrhizal infection rate of *A. mcphersonii* seedlings and the OTU composition of mycorrhizal communities. However, some OTUs were found in both types of environments. We discuss the effect of the disturbance on mycorrhizal communities associated with natural ectomycorrhizal *A. mcphersonii* seedlings, particularly in view of its application to ecological restoration of disturbed forest environments.

**Introduction**
Ecological restoration is a process of assisting the recovery of an ecosystem that is disturbed (Working Society for Ecological Restoration International Science and Policy 2004). Its objective is to re-initiate the ecosystem development in a dynamic that will lead to the reference state (Clewell and Aronson 2010). Two points are of major importance for ecological restoration: to re-establish mutualistic interactions between plants and soil microorganisms (Perry et al. 1989) and to promote exchanges between the surrounding landscape and the degraded ecosystem (Clewell and Aronson 2010).

Among mutualistic interactions between plants and soil microorganisms, mycorrhizal symbiosis, an association between a fungus and a plant root, is particularly important for ecosystem development (Wardle et al. 2004; Nara 2006). The fungus provides water and mineral nutrients to the plant and protects it against pathogens in exchange for carbon from photosynthesis (Smith and Read 2008; van der Heijden et al. 2015). Among the diverse mycorrhizal symbioses, the ectomycorrhizal one concerns most of the temperate and boreal forest trees and numerous abundant tropical forest trees (Wang and Qiu 2006). This symbiosis is mainly formed by Basidiomycetes and to a lesser extent by Ascomycetes, which are structured in more or less diversified, network-like communities in which a focus fungus associates with several trees (Selosse et al. 2006; Beiler et al. 2010), sometimes from different species, and a single tree associates with diverse ectomycorrhizal fungi species (Bahram et al. 2011). These common mycorrhizal networks promote ecosystem stability (Bascopme 2009; Simard et al. 2012). Ectomycorrhizal community diversity and composition spontaneously change over time (Henry et al. 2014) and are sensitive to environmental disturbances, by which they can be partially or totally destroyed (e.g. Jumpponen, Trappe and Cazares 2002; Lazaruk et al. 2005; Hartmann...
et al. 2012), and enter secondary or primary successions. Lazaruk et al. (2005) showed that environmental disturbances could lead to radical changes in mycorrhizal community composition. After such disturbances, the restoration of initial mycorrhizal interactions in degraded ecosystems requires flux of propagules (spores, sclerotia, or even hyphae) between natural and disturbed ecosystems. Propagules of ruderal fungi may come from formerly disturbed areas that are at the early stage of plant succession, but flux of propagules may be faster if some fungi are able to grow both in the surrounding intact natural forest and in the disturbed area where microclimatic and edaphic conditions are contrasted.

Some disturbances, such as forest clearing, lead to upper soil layer removal. Since strong differences in ectomycorrhizal community abundance, diversity and composition have been highlighted according to soil horizons in temperate forests (Dickie, Xu and Coide. 2002; Courty et al. 2008) and in boreal forests (Heinonsalo, Jorgensen and Sen 2001; Rosling et al. 2003; Tedersoo et al. 2003), this upper soil layer removal may be an additional obstacle for establishment of fungi coming from undisturbed areas. However, such studies are rare in tropical environments, where fungal ability to adapt to a disturbed environment or to a change in edaphic conditions is unknown.

Madagascar is a biodiversity hotspot prioritized for biodiversity protection programme settlement (Myers et al. 2000). On this island, the Ambatovy mining project exploits large nickel and cobalt deposits. This activity leads to forest clearing and soil removal over more than 1200 ha. The effect of environmental disturbance on ectomycorrhizal community composition and the ability of ectomycorrhizal fungi to grow both in the natural forest and in the disturbed environment were assessed in order to shed light on mycorrhizal fungi community resilience in this area. For this, ectomycorrhizal communities associated with Asteropeia mcphersonii seedlings spontaneously growing in natural forests and in open disturbed areas were compared.

**Material and methods**

**Study site**

The study site previously described in Henry et al. (2015) is in tropical rainforest in Madagascar, in the operational area of the Ambatovy nickel and cobalt mine.

**Experimental design**

We selected four sites where A. mcphersonii seedlings smaller than 10 cm were spontaneously growing both beside a track and in the adjacent natural forest (Figure 1).
Tracks were between 2 and 8 years old (Table 1). Site GPS coordinates are shown in Table 1. At each of the four sites, a plot was randomly selected in the forest and another plot was selected in the adjacent open disturbed area, about 3 m away from the previous one. In natural forests, the litter has a thickness of 15 cm and the whole root system of seedlings is in the litter. In open disturbed areas where the forest has been cleared and the upper soil layer removed, root systems are in a homogeneous mineral horizon. Disturbances are relatively old and the distance between forest and open disturbed area is small, so fungal dispersal is theoretically not a limiting factor for mycorrhizal communities associated with seedlings growing in open disturbed areas.

On each of the eight plots, all the root apices were harvested from 20 A. mcphersonii seedlings. All root tips were observed under a stereomicroscope at ×70 magnification. The root tip is considered ectomycorrhizal when surrounded by a fungal coat. For each plot, the ectomycorrhizal infection rate was defined as the number of ectomycorrhizal apices divided by the total number of apices. All apices were stored in CTAB (2% cetyltrimethylammonium bromide, 100 mM Tris–HCl pH 8.0, 1.4 M NaCl, 20 mM ethylenediaminetetraacetic acid) until molecular analysis.

Molecular analyses

Each root tip was subjected to DNA extraction using a modified protocol with the REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich, St Louis, MO, USA). The internal transcribed spacer (ITS) region including ITS1, ITS2 and 5.8S of the rDNA was amplified by polymerase chain reaction (PCR) using the primer pair ITS1F and ITS4 as in Henry et al. (2015). Samples that yielded multiple PCR products were submitted to a second PCR using the basidiomycete-specific primers ITS4B and ITS1F, also as in Henry et al. (2015). Genoscreen (Lille, France) sequenced both strands using the BigDye terminator v3.1 kit. Sequences were manually edited using Chromas pro v1.7.5 and merged into operational taxonomic units (OTUs), a proxy for molecular species, at a 97% similarity threshold on the common sequence section using the web server CD-HIT suite. Taxonomic affiliation of OTUs was obtained by a BLASTN search against the National Centre for Biotechnology Information (NCBI) database. One representative sequence for each OTU was deposited in GenBank (Accession numbers KT200520–KT200578).

A conservative taxonomic OTU assignment was conducted at the order level for Boletales and Agaricales (except for Tricholomataceae and Cortinariaceae), Sebacinales, Cantharellales, Corticales and Hymenochaetales. Thelephorales were reduced to their only known ectomycorrhizal family, Thelephoraceae. For Russulaceae, homologies in GenBank allowed affiliations at the family level. Two OTUs without clear taxonomic affiliation were named “unknown 1” and “unknown 2”. They were submitted to amplification and sequencing using primers ITS1F and TW13 to obtain more precise taxonomic affiliation with the 28S ribosomal region.

Data analyses

Statistical analyses were performed in the R environment (R Development Core Team 2011). Ectomycorrhizal infection rates were compared between communities growing on disturbed or forest plots using a Friedman rank sum test with unreplicated blocked data after an arcsine transformation (arcsin(√x)) of data. Each site was considered as a block. The relative abundance of each taxonomic group on each plot and the abundance on each plot of each OTU except singletons were represented by bar charts. To determine the effect of disturbance on OTU composition of A. mcphersonii seedling ectomycorrhizal communities and on the abundance of the main taxonomic groups, multiresponse permutation procedures (MRPPs) were performed using the ‘mrpp’ function of the ‘vegan’ package (Oksanen et al. 2013). The dissimilarity index used in these procedures is the Chao index, which takes into account rare species that the sampling effort did not track (Chao et al. 2004). Chao distances between ectomycorrhizal communities of each plot were represented by principal coordinates analysis (PCoA) on the two axes, which explains the most variability using the PCoA function of the ‘ape’ package (Paradis, Claude and Strimmer 2004). Bias in the presence of each of the main taxonomic groups on forest or disturbed plots was studied by the use of Fisher’s exact test.
The Friedman rank sum test showed that ectomycorrhizal infection rates were significantly lower in disturbed plots (mean = 36.5%, Table 1) than in forest plots (mean = 56%) \((p = 0.045)\). In total, 219 ectomycorrhizal roots yielded a tractable DNA sequence (Table 1). The main taxonomic groups represented are Russulaceae (74 mycorrhizae, 20 OTUs), Boletales (72 mycorrhizae, 18 OTUs) and Thelephoraceae (26 mycorrhizae, 14 OTUs; Figure 2). OTU 'unknown 1' is taxonomically close to the order Trechisporales. More than half of the mycorrhizae belong to an OTU that was found in only one plot. Similarly, two OTUs were found more than 20 times on a single plot, which indicates a high spatial aggregation/patchiness of the communities. Of the 34 OTUs represented by at least two mycorrhizae, fewer than half (15) were present on at least two plots. Only three of them were found in both forest and degraded environments (Figure 3).

A significant statistical effect \((p = 0.031)\) of the environment (natural forest versus open degraded area) on the mycorrhizal community composition in terms of OTUs has been found by MRPPs. PCoA showed a grouping of communities associated with the same environment, mostly on the principal axis (Figure 4). MRPPs did not highlight any significant effect of perturbation on the mycorrhizal community composition in terms of abundance \((p = 0.056)\) or OTU number \((p = 0.093)\) of the five main taxonomic groups (Russulaceae, Boletales, Thelephoraceae, Sebacinales and Agaricales). Fisher’s exact test did not show any effect of the disturbance on the presence of each of the five main taxonomic groups (see Supplemental data, Table S2).
whereas others prefer mineral horizons (Dickie, Xu and Koide 2002; Rosling et al. 2003; Genney, Anderson and Alexander 2006). Root systems of A. mcpersonii seedlings growing in the forest are in the litter, whereas those of seedlings growing in an open degraded environment are in mineral horizons, which may explain the differences observed. This hypothesis could be tested by a comparison between mycorrhizal communities associated with deep roots of older plants in natural forest that have grown enough to prospect the mineral horizon under the litter.

The microclimates experienced by A. mcpersonii seedlings differ greatly between natural forest and the open degraded environment, since solar radiation, air temperature, soil temperature and moisture are highly sensitive to canopy removal (Chen et al. 1999). Differences in soil moisture between the two types of environment can influence ectomycorrhizal community composition (Sims et al. 2007). Above the soil, the microclimate can have a great influence on the photosynthetic capacity of plants, which accumulate more carbon on deforested sites (Muraoka et al. 1997). As mycorrhizal community composition depends on the carbon provided by the trees (Saikkonen et al. 1999; Cullings et al. 2001; Smith and Read 2008), the microclimate can modify mycorrhizal community composition associated with A. mcpersonii seedlings.

In conclusion, environmental disturbance has a significant effect on mycorrhizal community composition in terms of OTUs, but few OTUs are capable of growing in both natural forest and an open degraded environment. These OTUs able to grow after the small-scale disturbance produced by track settlement will probably be able to grow after large-scale disturbances produced by mining and promote exchanges between the degraded environment and the reference ecosystem; exchanges that are essential for ecological restoration of the mining site. This capacity for exchanges could be preserved on the mining site by the conservation of vegetation patches containing ectomycorrhizal plants, or perhaps even by the transplantation to the degraded environment, at the beginning of ecological restoration, of natural ectomycorrhizal seedlings.

**Discussions**

There is no strict specificity of fungi to a given environment (natural forest versus open degraded area) as three OTUs were found in both environments. Some fungal species are therefore able to adapt to both environments.

The high abundance of mycorrhizae belonging to a single plot, which indicates a high spatial aggregation, makes it difficult to study one factor in the mycorrhizal community composition. This aggregation, which is quite common in ectomycorrhizal communities (Bahram, Peay and Tedersoo 2014), also limited the attempts of Tedersoo et al. (2003) to study the impact of the soil horizon in an Estonian mixed forest.

We show here an effect of the environmental disturbance on the composition of mycorrhizal communities growing on A. mcpersonii seedlings and on their ectomycorrhizal infection rate. Environmental disturbance could have destroyed a proportion of the ectomycorrhizal fungi (Lazaruk et al. 2005) and their dispersion therefore becomes the parameter that limits recolonization of the environment by fungi (Peay et al. 2007). In our system, theoretically fungal inoculum availability is not limiting, because disturbances occurred several years ago and seedlings from disturbed environments were harvested near A. mcpersonii adults and also near natural forest seedlings. The differences in terms of OTU composition that we observed between natural forest communities and open perturbed environment communities are therefore probably due to two environmental parameters: soil composition and microclimate.

Mycorrhizal communities are structured by the different ecological niches that exist on a small scale in the soil (Buée et al. 2007; Courty et al. 2008). For example, some species prefer litter or organic horizons, whereas others prefer mineral horizons (Dickie, Xu and Koide 2002; Rosling et al. 2003; Genney, Anderson and Alexander 2006). Root systems of A. mcpersonii seedlings growing in the forest are in the litter, whereas those of seedlings growing in an open degraded environment are in mineral horizons, which may explain the differences observed. This hypothesis could be tested by a comparison between mycorrhizal communities associated with deep roots of older plants in natural forest that have grown enough to prospect the mineral horizon under the litter.

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