



Diversity of foliar endophytic ascomycetes in the endemic Corsican pine forests

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ABSTRACT

Plant leaves host species rich communities of foliar endophytic fungi (FEF). Compared to the other compartments of the plant microbiome, FEF diversity is poorly known. Here we document the communities of FEF associated with the endemic Corsican pine *Pinus nigra* subsp. *laricio* at three sites across its natural range and examine the effect of forest site, tree age and light exposure on FEF composition. Metabarcoding using next-generation sequencing provided 8,243,608 Ascomycota ITS2 sequences clustered into 642 FEF operational taxonomic units (OTUs). FEF communities did not differ in species richness across sites and tree cohorts, but differed in composition among forest patches and according to tree age. FEF community composition did not correlate with needle location (shade vs full light). Results are robust against the various options of the bioinformatic pipeline specifically developed. This study provides the first picture of FEF diversity in a Mediterranean island and underlines the complementarity of forest massifs for fungal conservation.

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1. Introduction

Understanding the full complexity of plants as chimeric structures involving micro and macro-organisms is a critical issue in ecology (Partida-Martinez and Heil, 2011; Vandenkoornhuys et al.,

2015). In particular, while leaves support the entire food web in forest ecosystems, they also represent a key location for interactions between plants and their microbiomes. The phyllosphere microbiome — the community of all microorganisms living on the surface of leaves (epiphytes) or inside (endophytes) — is mainly composed of fungi and bacteria (Christian et al., 2015; Hardoim et al., 2015). These microorganisms are an essential component of the plant extended phenotype (Partida-Martinez and Heil, 2011). They influence plant growth and survival (Friesen et al., 2011; Porras-Alfaro and Bayman, 2011), and mediate plant functional traits (Friesen et al., 2011).

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Endophytic microorganisms *sensu lato* encompass mutualistic (endophytes *sensu stricto*), pathogenic and commensal bacteria and fungi (Hardoim et al., 2015). Foliar endophytic fungi (FEF) include Basidio- and Chytridiomycetes but are mainly composed of Ascomycota (Table 1, Kembel and Mueller, 2014). Despite their ecological importance, very little is known about the ecology of FEF. In our study, we consider as endophytic all fungi that are present inside apparently sound leaf tissues, without requiring a measurable effect on plant phenotype (endophyte *sensu lato*; see Hardoim et al., 2015).

The functioning of plants and their associated microorganisms — the holobiont — depends on FEF community composition (Hardoim et al., 2015; Vandenkoornhuysen et al., 2015). FEF provide the plant with protection against various pathogens, herbivory and abiotic stress such as drought (Porras-Alfaro and Bayman, 2011; Christian et al., 2015). For example, the presence of the fungal endophyte *Colletotrichum tropicale* reduces the defoliation of *Cucumis sativus* by leaf-cutting ants (Estrada et al., 2013). These positive interactions between plants and FEF have inspired a wide range of laboratory tests in a quest for new biologically active molecules (e.g. for antitumoral, antibacterial, fungicide activities; reviewed in Kaul et al., 2012 and Mishra et al., 2014) and ambitious industrial developments (e.g. insecticide production; Mousa and Raizada, 2013). In ecology, endophytes are promising model organisms to get a more comprehensive understanding of plant functioning (Vandenkoornhuysen et al., 2015) and to test ecological concepts (Meyer and Leveau, 2012). Moreover, they represent a considerable and largely under-explored microbiota critical for ecosystem functioning (Friesen et al., 2011; Yuan and Chen, 2014; Hardoim et al., 2015).

Previous work has provided some insights into the determinants and the scale of variations in FEF community composition based on culturing mycelium isolates and DNA sequencing, including next-generation sequencing. In a multi-host study (51 host species) in a tropical rainforest, Kembel and Mueller (2014) found that the taxonomic identity of the trees might explain more than half of the variation in composition of FEF communities. Within a tree species, other factors are likely to act, including biotic factors such as host genotype (Bálint et al., 2013, 2015 on populations of *Populus balsamifera* across USA). Abiotic factors such as altitude (Siddique and Unterseher, 2016) or temperature, as determined by slope orientation, have also been shown to correlate with the composition of FEF communities (Koide et al., 2017). Within individual trees, the composition of FEF communities has been shown to be structured according to (i) the distance between leaves, as FEF communities are more similar between spatially close leaves than between more distant ones (Cordier et al., 2012, on *Fagus sylvatica*), and (ii) the age of leaves, with old leaves displaying higher FEF diversity than young ones (Helander et al., 1994; Millberg et al., 2015). So far, only a few studies (Table 1) have documented FEF diversity patterns and community variations at different scales, from leaves to forests at the regional scale (Millberg et al., 2015; Oono et al., 2015, 2017; Nguyen et al., 2016; Koide et al., 2017).

Corsican pine (*Pinus nigra* subsp. *laricio*) is the emblematic tree species of the fourth largest Mediterranean island. Corsican pine forests are listed among the ecosystems whose conservation is a priority in the European Union Council Directive 92/43/EEC. This Corso-Calabrian-Sicilian-endemic pine dominates mountain landscapes in Corsica between 1200 and 1800 m of altitude (Jeanmonod and Gamisans, 2007). During the last two decades, *P. nigra* subsp. *laricio* forests have suffered large wildfires of anthropogenic origin and intensive management of forest timber that together dramatically affected their structure and the associated diversity (e.g. birds, Moneglia et al., 2009). After a long-history of high human

pressure on this ecosystem, only a few old-growth stands remain across the Corsica island. The current situation raises conservation concerns as the oldest trees of this very long-lived species host the endemic Corsican nuthatch (Moneglia et al., 2009), but also a remarkable diversity of saproxylic fungi associated with old-growth forest, some of which may be relicts from the last glaciation (Norstedt et al., 2001). However, we have no prediction as to whether these old trees also host a remarkable microbiome in terms of foliar endophytic fungi.

The main objective of our study was to explore the diversity of ascomycete FEF associated with the Corsican pine and the variation in FEF community composition across the lifetime and natural range in Corsica of this long-lived pine species. The available literature prompted us to test (i) whether variation in light exposure and host age correlates with variation in FEF community species richness and diversity, (ii) whether old trees are reservoirs of FEF species, hosting a higher species richness than young trees, and (iii) given the narrow natural range of Corsican pine and the high dispersion capabilities of Ascomycota, whether FEF communities were homogeneous among sites at the same altitude. To answer these questions, we devised a sampling protocol in three old-growth stands distributed across the central mountains of the island and at roughly the same altitude, from the northernmost (Asco) to the southernmost (Bavella) location of its natural range in Corsica. At each of these three sites, we selected individuals varying in age from saplings to several-hundred-year-old trees. For each of them, we explored the composition of FEF communities at three levels of light exposure corresponding to three elevation levels in the tree (Fig. 1A), via a metabarcoding approach using next-generation sequencing of environmental samples.

2. Materials and methods

2.1. Sampling procedure

In Corsica, the Corsican black pine (*P. nigra* subsp. *laricio*) forms around 16,000 ha of pure woodlands (Moneglia et al., 2009). Across the island, this very long-lived tree species persists in a few old-growth stands characterized by unevenly aged individuals (Gamisans and Marzocchi, 1996; Norstedt et al., 2001). In these forests, scattered several-hundred-year-old individuals, some over 400 years-old (Norstedt et al., 2001), dominate a dense understory composed of various cohorts of pines, from deep shade seedlings to mature canopy trees.

In May 2013, we selected three old-growth stands of Corsican pines (forests of Asco [42.3991°N; 8.9187°E; 1520 m a.s.l.], Verghello [42.1935°N; 9.0883°E; 1390 m a.s.l.] and Bavella [41.7928°N; 9.2199°E; 1230 m a.s.l.]; Asco and Bavella are located 70 km apart, Fig. 1A). In each stand, we selected nine individuals including three >250 y-old trees (Fig. 1B, hereafter called old, reaching on average 22, 40 and 18 m high at Asco, Verghello and Bavella, respectively), three 70–130 y-old trees (reaching approximately the same height as the older trees) and three 10–15 y-old trees (young, Fig. 1A), all located at a minimum distance of 50 m from each other. None of the sampled trees was in the shadow of another tree. For each of the 27 selected trees, 1 y-old needles were collected in three contrasting light exposure conditions: (i) full shade understory branches at low elevation in the tree, hereafter called “low”, (ii) tree half-height branches, called “intermediate” and (iii) full light canopy branches, called “high”. In total, we collected 81 needle samples. Each sample consisted of 30 needles collected on three distinct branches on one year-old shoots, devoid of visible pathogen damage or lichen colonization.

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