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Phylogenetic analyses of eurotiomycetous endophytes reveal their close affinities to Chaetothyriales, Eurotiales, and a new order – Phaeomoniellales

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ABSTRACT

Symbiotic fungi living in plants as endophytes, and in lichens as endolichenic fungi, cause no apparent symptoms to their hosts. They are ubiquitous, ecologically important, hyperdiverse, and represent a rich source of secondary compounds for new pharmaceutical and biocontrol products. Due in part to the lack of visible reproductive structures and other distinctive phenotypic traits for many species, the diversity and phylogenetic affiliations of these cryptic fungi are often poorly known. The goal of this study was to determine the phylogenetic placement of representative endophytes within the Eurotiomycetes (Pezizomycotina, Ascomycota), one of the most diverse and evolutionarily dynamic fungal classes, and to use that information to infer processes of macroevolution in trophic modes. Sequences of a single locus marker spanning the nuclear ribosomal internal transcribed spacer region (nrITS) and 600 base pairs at the 5' end of the nuclear ribosomal large subunit (nrLSU) were obtained from previous studies of >6000 endophytic and endolichenic fungi from diverse biogeographic locations and hosts. We conducted phylum-wide phylogenetic searches using this marker to determine which fungal strains belonged to Eurotiomycetes and the results were used as the basis for a class-wide, seven-locus phylogenetic study focusing on endophytic and endolichenic Eurotiomycetes. Our cumulative supermatrix-based analyses revealed that representative endophytes within Eurotiomycetes are distributed in three main clades: Eurotiales, Chaetothyriales and *Phaeomoniellales* ord. nov., a clade that had not yet been described formally. This new order, described herein, is sister to the clade including Verrucariales and Chaetothyriales. It appears to consist mainly of endophytes and plant pathogens. Morphological characters of endophytic *Phaeomoniellales* resemble those of the pathogenic genus *Phaeomoniella*. This study highlights the capacity of endophytic and endolichenic fungi to expand our understanding of the ecological modes associated with particular clades, and provides a first estimation of their phylogenetic relationships in the Eurotiomycetes.

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1. Introduction

All plant species sampled to date harbor endophytic fungi, which are fungal symbionts inhabiting living tissues such as roots,

leaves and stems without causing obvious symptoms (Rodríguez et al., 2009; Saikkonen et al., 1998). Many studies have reported the extremely high biodiversity of fungal endophytes in above-ground tissues of plants (e.g., Arnold and Lutzoni, 2007; Lodge et al., 1996; Zimmerman and Vitousek, 2012). Endophyte species richness is predominately found within the subphylum Pezizomycotina (Ascomycota) encompassing the majority of the filamentous ascomycetes (see Arnold et al., 2009; U'Ren et al., 2010, 2012). In addition to important roles in plant physiology and ecology (Ernst et al., 2003; Hubbard et al., 2014; Rodríguez et al., 2008), the potential of endophytes as a resource for biological control (Arnold et al., 2003; Backman and Sikora, 2008) and

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pharmaceutical products (e.g., Stierle et al., 1993) is also widely acknowledged. Despite their ubiquity and potential importance, studies of endophytes have focused primarily on diversity at the species level (e.g., Gazis et al., 2011; Ovaskainen et al., 2010; but see U'Ren et al., 2012), with relatively little information available so far regarding their phylogenetic relationships or broader contributions to the fungal tree of life (but see Arnold et al., 2009; Qadri et al., 2014; Spatafora et al., 2007).

Much like endophytes in plants, endolichenic fungi are endophyte-like symbionts that live inside apparently healthy lichen thalli, primarily in association with algal and/or cyanobacterial cells (Arnold et al., 2009). Endolichenic fungi are distinct from mycobionts, which make up the lichen thallus, and from lichenicolous fungi, the reproductive structures of which (sexual and/or asexual) can be often observed on living lichens (Arnold et al., 2009). They also are largely distinct at the species level from foliar endophytic fungal communities in vascular plants, but are frequently shared with co-occurring bryophytes (even when not growing in close physical proximity; see U'Ren et al., 2010, 2012). U'Ren et al. (2010, 2012, 2014) also reported that endolichenic fungal communities are abundant and diverse.

Relationships of endophytic and endolichenic fungi (hereafter, collectively referred to as fungal endophytes or endophytes) have been explored only once in a phylum-wide phylogenetic framework (Arnold et al., 2009). Since that time many studies have documented additional endophyte taxa (e.g., Del Olmo-Ruiz and Arnold, 2014; Gazis and Chaverri, 2010; Larkin et al., 2012; U'Ren et al., 2012, 2014; Zimmerman and Vitousek, 2012), and knowledge of the Pezizomycotina tree of life has advanced substantively (e.g., Gazis et al., 2012; Prieto et al., 2013; Schoch et al., 2009), prompting new exploration of the phylogenetic relationships of endophytes in a phylogenetically broad and robust context.

The kingdom Fungi is one of the most diverse groups of eukaryotes on earth (Blackwell, 2011; Hibbett and Taylor, 2013). Although the total species richness of fungi is estimated to be up to 5.1 million (Blackwell, 2011), only about 100,000 have been described (Blackwell, 2011; Kirk et al., 2008). Many fungal species are believed to be living cryptically, in symbiosis with other organisms such as plants and insects (Blackwell, 2011). Although culture-independent methods and advancements in high-throughput sequencing technology have greatly hastened the discovery of fungal biodiversity, the phylogenetic placement and taxonomy of most fungal endophytes have not been explored. This is in part because (1) some endophytes are not culturable (e.g., Arnold et al., 2007; Impullitti and Malvick, 2013; Pancher et al., 2012); (2) even when culturable, many fungal endophytes do not form *in vitro* the morphological structures that are traditionally used in fungal taxonomy (Petrini and Petrini, 1985); and (3) most culture-independent studies of fungi rely on the nuclear ribosomal internal transcribed spacer region (nrITS), which is not amenable to broad-scale phylogenetic analysis, or use short reads that provide limited resolving power (Lindner and Banik, 2011; Porter and Golding, 2011). Moreover, (4) the great majority of endophyte species have not been described; thus, even if many sequences of endophytes have been deposited in GenBank, they usually provide limited taxonomic information (e.g., see Gazis et al., 2012; Nilsson et al., 2014; U'Ren et al., 2009). These issues lead to uncertainty with regard to how best to delimit species and other taxonomic groups for endophytes and related fungi, limiting ecological inferences and diminishing our ability to address evolutionary questions.

The class Eurotiomycetes (Pezizomycotina, Ascomycota) includes species with highly varied metabolic abilities, many of which are important in human health and sustainability (e.g., Geiser et al., 2006). The order Eurotiales includes mainly saprotrophic genera like *Aspergillus* and *Penicillium*, but also animal-as-

sociated genera such as *Trichophyton* and *Onygena*. The orders Pyrenulales and Verrucariales include some lichen mycobionts. Extreme environments, such as xeric rock surfaces, are colonized by some Chaetothyriales, and by members of the subclass Chaetothyrionmycetidae (containing Chaetothyriales, Pyrenulales and Verrucariales) in general (Geiser et al., 2006; Gueidan et al., 2008). Several groups also contain opportunistic human pathogens that can switch from saprotrophic to pathogenic lifestyles (e.g., Barker et al., 2007; Hohl and Feldmesser, 2007).

In addition to interacting with dead plant tissue and living animals, many Eurotiomycetes associate closely with living plants and lichens. For example, *Phaeoconiella chlamydospora* is a causal agent of Petri disease of grapevine (Crous and Gams, 2000) and *Elaphomyces* forms ectomycorrhizal associations with trees (Castellano et al., 2012). Some species occur as lichenicolous fungi (e.g., Diederich et al., 2013; Réblová et al., 2013), and others – especially *Aspergillus* and *Penicillium* – can be isolated as endophytes (see Arnold et al., 2009; Naik et al., 2009; Peterson et al., 2005; Sandberg et al., 2014; U'Ren et al., 2012; Vega et al., 2010).

Here, we resolve the phylogenetic and taxonomic affinities of representative endophytes within Eurotiomycetes, and explore for the first time the origin of endophytism and endolichenism within this diverse class. Previous culture-based work, which characterized endophytic and endolichenic fungi from diverse biomes across North America using sequence data from the nrITS and a portion of the adjacent nuclear ribosomal large subunit (nrLSU) (i.e., Arnold et al., 2009; U'Ren et al., 2010, 2012), suggested the placement of a number of strains within Eurotiomycetes based on BLAST. We used these data as the basis for a seven-locus phylogenetic approach to address the following questions: (1) What is the evolutionary history of endophytes within Eurotiomycetes? (2) How do endophytes fit within the current classification of Eurotiomycetes? (3) What are the geographical, ecological and, phenotypic features of these endophytic taxa? Further, (4) we tested the reliability of using the nr5.8S + LSU from our target nrITS-LSU locus to infer relationships of unknown endophytes within the broad scope of the Pezizomycotina, as a complementary approach to BLAST analysis with nrITS (the fungal DNA barcode; Schoch et al., 2012)). Our results reveal an order (Phaeoconiellales) that was observed but not described formally in previous work within Eurotiomycetes (Gueidan et al., 2014; Rossman et al., 2010). Phaeoconiellales appears to be composed mainly of endophytic fungi and plant pathogens. We use data from the nrLSU to infer the most comprehensive phylogenetic tree to date for this new order of fungi, and draw from metadata provided by ecological studies to evaluate major trends in their geographic- and host affiliations.

2. Materials and methods

2.1. Endophyte isolation, DNA extraction, and nrITS-LSU sequence acquisition

We used a collection of 6521 fungal cultures isolated through studies examining the abundance, diversity, ecology, and evolution of fungal endophytes (i.e., Arnold and Lutzoni, 2007; Arnold et al., 2009; Arnold, unpubl. data; Higgins et al., 2007; Hoffman and Arnold, 2008; U'Ren et al., 2010, 2012). These endophytes were collected in 2003–2009 from six major lineages of land plants (bryophytes, lycophytes, monilophytes, gymnosperms, monocots, and eudicots; Fig. 1A) and three functional groups of lichens (epiphytic, saxicolous, and terricolous/muscicolous; Fig. 1B). These samples represent diverse geographic and biogeographic provinces, as described in full by Arnold and Lutzoni (2007), Arnold et al. (2009), Higgins et al. (2007), Hoffman and Arnold (2008) and

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