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Interspecific variability of class II hydrophobin GEO1 in the genus *Geosmithia*

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ABSTRACT

The genus *Geosmithia* Pitt (*Ascomycota: Hypocreales*) comprises cosmopolite fungi living in the galleries built by phloeophagous insects. Following the characterization in *Geosmithia* species 5 of the class II hydrophobin GEO1 and of the corresponding gene, the presence of the *geo1* gene was investigated in 26 strains derived from different host plants and geographic locations and representing the whole phylogenetic diversity of the genus. The *geo1* gene was detected in all the species tested where it maintained the general organization shown in *Geosmithia* species 5, comprising three exons and two introns. Size variations were found in both introns and in the first exon, the latter being due to the presence of an intragenic tandem repeat sequence corresponding to a stretch of glycine residues in the deduced proteins. At the amino acid level the deduced proteins had 44.6 % identity and no major differences in the biochemical parameters (pI, GRAVY index, hydropathy plots) were found. GEO1 release in the fungal culture medium was also assessed by turbidimetric assay and SDS-PAGE, and showed high variability between species. The phylogeny based on the *geo1* sequences did not correspond to that generated from a neutral marker (ITS rDNA), suggesting that sequence similarities could be influenced by other factors than phylogenetic relatedness, such as the intimacy of the symbiosis with insect vectors. The hypothesis of a strong selection pressure on the *geo1* gene was sustained by the low values (<1) of non synonymous to synonymous nucleotide substitutions ratios (*Ka/Ks*), which suggest that purifying selection might act on this gene. These results are compatible with either a birth-and-death evolution scenario or horizontal transfer of the gene between *Geosmithia* species.

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Introduction

The monophyletic genus *Geosmithia* Pitt (*Ascomycota: Hypocreales*) comprises 32 published species of mitosporic fungi mostly associated with phloem-feeding bark beetles belonging to the Scolytids and Bostrichis (Kolařík *et al.* 2004, 2005, 2007, 2008, 2011; Kolařík & Kirkendall 2010; Kolařík 2012; Kolařík & Jankowiak 2013). *Geosmithia* fungi are mainly insect-associated but can also live on various plant substrates without relation to insects, soil, foodstuffs and as true plant endophytes (Kolařík *et al.* 2004, 2008; Kolařík & Jankowiak 2013; McPherson *et al.* 2013). Two primary ambrosia fungi, *Geosmithia eupagioceri* and *Geosmithia microcorthyli*, associated with beetle species in Costa Rica, have also been described (Kolařík & Kirkendall 2010). Only one phytopathogenic species has been identified so far, *Geosmithia morbida*, the causal agent of thousand-canker disease of black walnut in USA (Kolařík *et al.* 2011). However, Čížková *et al.* (2005) reported an inhibitory effect on stem and root elongation in oak plants for the species *Geosmithia pallida* and *Geosmithia langdonii*.

Geosmithia are abundant associates of numerous subcortical insects worldwide, and there is growing evidence that this association is consistent and evolutionarily stable. The most convincing proof is the presence of true ambrosia species, of the bark beetle-vectored phytopathogenic species *G. morbida* and the fact that some species are specialists restricted to several insect vectors and host plants over large geographical areas (Kolařík *et al.* 2008; Kolařík & Jankowiak 2013). Insect vectors infest hardwoods and conifers and are widespread in central Europe and in the tropics of America, Asia and Australia. The advantage for the beetles of the association with *Geosmithia* is still unclear, except in the case of ambrosia species; fungi can provide food for the insects or affect their fitness through the production of secondary metabolites i.e. hydroxylated anthraquinones, that could inhibit detrimental microbes for the host beetle as well as acting as repellents towards the beetle's predators (Stodůlková *et al.* 2009). Kolařík & Kirkendall (2010) have proposed that the association of fungi with phloeophagous bark beetles was evolutionarily ancestral, followed by at least three independent shifts to obligate association with ambrosia beetles and then by fundamental morphological adaptations.

Bettini *et al.* (2012) have recently reported on the isolation in *Geosmithia* species 5 strain IVV7 of a new class II hydrophobin, called GEO1, and of the corresponding gene. Hydrophobins are small proteins produced by filamentous fungi whose main characteristic is the ability to assemble at the hydrophilic/hydrophobic interfaces forming an amphipathic membrane (Sunde *et al.* 2008). They have been divided in two classes based on their solubility, hydropathy patterns and amino acid sequences: class I hydrophobins are produced by ascomycetes and basidiomycetes, while class II hydrophobins are produced only by ascomycetes (Whiteford & Spanu 2002; Linder *et al.* 2005). Hydrophobins are involved in fungal development and in the interaction between fungi and their hosts, being in some cases pathogenicity factors. In particular, they can mediate the attachment of fungi to hydrophobic surfaces, such as plant cuticle, lignin, or insect exoskeleton (Wösten *et al.* 1994; Temple & Horgen 2000; Zhang *et al.* 2011).

Conidia produced by *Geosmithia* are dry and hydrophobic as in airborne fungi (Kolařík *et al.* 2008), at variance with other entomochoric species, such as the *Ophiostoma*, which produce sticky conidia. The GEO1 hydrophobin could therefore favour the dissemination of the fungus by virtue of the hydrophobicity conferred to the conidia, which would allow it to establish hydrophobic interactions between the chitinous exoskeleton of the insect vectors and the conidia themselves (Temple & Horgen 2000; Zhang *et al.* 2011).

With the aim of studying the variability of GEO1 in *Geosmithia* species, in the present paper we describe the characterization of the *geo1* nucleotide sequences and of the deduced proteins in 26 species representing the phylogenetic diversity of the genus, isolated from different host plants and geographic locations.

Materials and methods

Fungal strains and culture

The *Geosmithia* strains representing 26 different species used in this study (Table 1) were isolated from insects as described (Kolařík *et al.* 2007, 2008; Kolařík & Jankowiak 2013) and maintained on Potato Dextrose Agar medium (BD Difco™). Plates were incubated in the dark at 24 ± 1 °C. For liquid culture, an agar plug was transferred to 100 ml flasks containing 20 ml of Takai medium modified as described in Scala *et al.* (1994). Flasks were wrapped in aluminium foil and incubated on a rotary shaker at 100 rpm at 24 ± 1 °C. To recover the mycelium cultures were centrifuged at 2500 rcf for 20 min at room temperature and pellets were stored at −20 °C.

DNA extraction and Polymerase Chain Reaction (PCR)

Genomic DNA extraction from mycelium was carried out with the NucleoSpin Plant II kit (Macherey–Nagel GmbH & Co. KG) following the manufacturer's instructions. DNA concentration was evaluated with a Qubit® 2.0 fluorometer (Invitrogen by Life Technologies), and PCR amplifications were carried out on 50 ng of DNA as described (Bettini *et al.* 2012). For the amplification of the *geo1* gene (GenBank accession no. JQ042234) the following primers were used: 5′-AAATGAAGTCCTTTGCCATCA-3′ (forward) and 5′-GAGAGTAACCCGGCACTTAGC-3′ (reverse).

DNA sequencing and bioinformatic analysis

Sequencing of the amplified fragments was carried out by Eurofins MWG Operon (Ebersberg, Germany), on either purified PCR products or on bands extracted from agarose gels with the NucleoSpin Gel and PCR Cleanup kit (Macherey–Nagel GmbH & Co. KG).

Sequences were aligned with MUSCLE (Edgar 2004). Nucleotide diversity (π), DNA polymorphism and the ratio of the number of non synonymous (*Ka*) to synonymous (*Ks*) substitutions for all pairwise comparisons of the 27 sequences coding for the premature protein, including that of our reference strain *Geosmithia* sp. 5 IVV7, were calculated with DnaSP