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Adaptive traits of bark and ambrosia beetle-associated fungi

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ABSTRACT

A phenotype is the expression of interactions between species genotype and environment. We quantified the contributions of ecological and phylogenetic associations to phenotypic variation in *Geosmithia* fungi. *Geosmithia* are symbiotic beetle-associated saprotrophs with a range of life histories and host specificities, including obligate nutritional beetle mutualists (ambrosia fungi) and phytopathogens. We hypothesized that: (1) species phenotypes are better explained by their ecology than by their phylogenetic relationships; (2) niche specialization was accompanied by enzymatic capability losses; and (3) ambrosia *Geosmithia* species have higher nutritional quality and antibiotic capabilities than species with facultative symbioses. Our results confirmed that long-term co-evolved specialists have reduced metabolic breadth in comparison to generalists. Phytopathogenic *G. morbida* produces unique enzyme suites with affinity to ligno-cellulose. Mycelia of ambrosia fungi contain large amounts of oleic fatty acid with nutritive and possibly allelopathic function. Overall, our results indicate that *Geosmithia* ecology have greater effect on species phenotype than their phylogenetic relationships.

1. Introduction

Bark beetles primarily feed on plant tissues, but fungi are often important nutritional components throughout their life cycle (Harrington, 2005), and these fungi greatly affect the beetles' subcortical niche (Six, 2013). Fungal-beetle relationships can be described from several perspectives, i.e., according to the strength of the statistical frequency of association, according to the direction of symbiosis (mutual benefit or parasitism) and whether co-evolution has occurred between the beetle vectors and the fungi. Many hypotheses have been proposed about the influence of the most frequent fungal associates on the beetles, including pathogenic effects on the plant host, degradation of tree-produced defence compounds, increased stress tolerance or production of antibiotics (Hofstetter et al., 2015). However, statistical association does not imply causality, and even less is known about whether any reciprocal co-evolution has taken place. Similarly, some fungal associates of beetles are phytopathogenic, for example *Ophiostoma novo-ulmi*, *Leptographium wageneri* (Kirisits, 2004) and *Raffaelea lauricola* (Ploetz et al., 2013), but their effects on the fitness of the vectoring beetles have not been assessed. It is likely that the fungal associates influence the nutritional ecology of the beetles, which calls for comprehensive studies of their metabolic capabilities. Whereas the relationships between bark beetles and fungi are often facultative, associations between ambrosia beetles and their fungi are defined by obligate relationships. Ambrosia fungi are cultivated by ambrosia beetles in their galleries situated in the xylem and are typically the sole source of beetle nutrition. Thus ambrosia fungi are essential, mutually beneficial, and co-evolved.

Nutritional relationships between mutualistic fungi and their vectors drive symbioses. Yet we are only beginning to understand the adaptive evolutionary changes that arise from fungus-beetle associations. For example, mutualistic fungi accumulate nitrogen in galleries which greatly increases the rate of beetle development (Ayres et al., 2000). However, the specific nutritional ecology of

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ambrosia fungi has rarely been studied (Kim et al., 2011; De Fine Licht and Biedermann, 2012). Fungi also provide sterols needed by the beetles as hormone precursors (e.g. ecdysone) and as building blocks of the cell membrane (Clayton, 1963). Kok et al. (1979) suggested that ambrosia beetles from the genus *Xyleborus* cannot develop without the fungal sterol ergosterol. However, Bentz and Six (2006) did not find any relation in the closeness of the beetle/fungus association and the quantity of ergosterol in fungal dry weight. Thus, the importance of ergosterol content remains ambiguous. Most ambrosia fungi do not degrade the structural polymers of plant tissues, such as cellulose and lignin (Kim et al., 2011; De Fine Licht and Biedermann, 2012), yet there are exceptions such as basidiomycete ambrosia fungi (Kasson et al., 2016). In summary, fundamental questions about the beetle-fungus nutritional ecology remain unanswered, including which fungal traits, such as nutritional value (e.g. ergosterol and lipid content), allelopathy or enzymatic activities, are under selective pressure in relation to the association between transmitted fungi and their vectors.

Species in the fungal genus *Geosmithia* (Ascomycota: Hypocreales) are widespread and abundant associates of subcortical insects, particularly bark beetles (Curculionidae: Scolytinae) and auger beetles (Bostrichidae) (reviewed in Kolařík et al., 2017). The majority of *Geosmithia* species are frequently associated generalists, vectored by a broad range of insects feeding on a variety of mainly hardwood plants and sometimes occurring outside of the bark beetle galleries (e.g., as endophytes or soil fungi – Kolařík et al., 2017). However, some species have highly specific niches, such as the Pinaceae-specialists which have never been found outside of this habitat (Kolařík and Jankowiak, 2013; Jankowiak et al., 2014; Kolařík et al., 2017). Angiosperm tree-dwelling specialists are usually specific to particular tree genera: *G. ulmacea* to *Ulmus*, (Pepori et al.,. 2015), *G.* sp. 8 to *Quercus*, *G.* sp. 12 to *Fraxinus* (Kolařík et al., 2008) and *G. morbida* to *Juglans* (Kolařík et al., 2011). *Geosmithia morbida* is a pathogenic species which causes Thousand Cankers Disease in black walnut (*Juglans nigra*) (Tisserat et al., 2009; Kolařík et al., 2011). It has impacted black walnut across the USA and has recently been found in Europe. The mechanisms of its pathogenicity remain unknown despite research efforts (Schuelke et al., 2017). Three independent origins of nutritional ambrosia fungi have been documented in *Geosmithia*. These include *G. microcorthyli*, *G. eupagioceri* (Kolařík and Kirkendall, 2010) and likely also *G. cnesini* (Kolařík et al., 2015). *Geosmithia rufescens*, which accompanies the ambrosial *G. eupagioceri* and *G. cnesini* in ambrosia beetle galleries, is an auxiliary ambrosia fungus (Kolařík and Kirkendall, 2010).

Comparative eco-physiology was successfully used in tracing adaptive traits in fungal symbionts of ants (De Fine Licht, Schiøtt et al., 2010) and in a fungal pathogen of bats (Chaturvedi et al., 2018). The aims of this project were to use comparative eco-physiology, and to attribute functional trait variation among *Geosmithia* species to ecological or phylogenetic components. This separation further enabled us to identify traits that are under selective pressure, and their importance in the formation of particular symbiotic associations.

In this study, we analyzed the enzyme profiles, biochemical composition and antibiotic capabilities of *Geosmithia* species. Enzymatic analyses outline the abilities of fungi to metabolize nutrients in plant tissues and thus make them accessible to the beetles through mycophagy. Biochemical composition approximates the nutritive value of fungal mycelia. Antibiotic capabilities reflect the potential of fungi to protect beetle galleries against microbial competitors and enemies. Our hypotheses were that: (1) species phenotypes are better explained by their ecology than by their phylogenetic relationships; (2) niche specialization was accompanied by enzymatic capability losses; and (3) *Geosmithia* mutualists have higher nutritional quality and antibiotic capabilities than species with facultative symbioses.

2. Materials and methods

2.1. Fungal material and cultivation

Twenty-three *Geosmithia* species were selected (Table 1) to represent diverse individual ecological strategies and phylogenetic lineages. These strains had been kept in culture for several years (1–10 y); this has no effect on their metabolic capabilities, however (Veselská and Kolařík, in press). We define generalists as those found on a wide range of plant families, primarily angiosperm trees. Accordingly, we define specialists as those restricted to a single plant genus or family, including the pathogen *G. morbida*, and ambrosia fungi specific to a single ambrosia beetle taxon. These strains are deposited in the Culture Collection of Fungi (CCF) or at the Institute of Microbiology of the Czech Academy of Sciences. The default growth medium was 2% malt extract agar (malt extract 20 g, glucose 20 g, peptone 1 g, 1 l of distilled H_2O), unless noted otherwise.

2.2. Phylogenetic analyses

All isolates were identified to the species level based on ITS rDNA sequences or alternative gene markers described in the original publications (Table 1). A phylogeny was constructed based on partial sequences of elongation factor 1α (*TEF-1*α) and the second largest subunit of the RNA polymerase II gene (*RPB2*). Both genes were amplified and sequenced according to Kolařík et al. (2017). Sequence alignments were obtained using MAFFT 6 (Katoh and Toh, 2008) (see Appendix 1 for dataset). Bayesian phylogenetic analyses were performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). A metropolis-coupled Markov chain Monte Carlo search algorithm with 2,000,000 generations was used. Burn-in was determined using Tracer 1.4 (http://tree.bio.ed.ac.uk/software/tracer) and discarded. Evolutionary models (TN93 + G) were determined by using MEGA 5.05 (Tamura et al., 2011).

2.3. Enzymatic analysis

2.3.1. Analysis of extracellular enzyme activities

Enzymes were extracted from cultures growing on a semi-natural medium (SNM) for one month. The SNM, developed during the course of our study, was composed of minced bark beetle adults (*Ips typographus*) and phloem of lime (*Tilia cordata*) and pine (*Pinus sylvestris*) as these trees are a common substrate for *Geosmithia*. Minced beetles and phloem were air dried, sterilized by autoclaving and mixed in a ratio of 1:23:23 (beetles: lime phloem: pine phloem). Glass Petri dishes with the sterile dried SNM (6 g per dish) were moistened by an inoculation solution (1 g of Bacto-pepton and 1 g of Bacto-yeast extract, Difco, in 1 l of distilled water) containing conidia ($3.2–4.5 \times 10^6$ conidia/ml) to attain the final moisture level of 75%. Inoculated SNM dishes were sealed with adhesive tape at four points for safe manipulation, deposited in open plastic bags to retain the moisture and air flow, and cultivated for one month. After that, the entire content of each Petri dish was put in 50-ml tubes, mixed with 30 ml of 50 mM sodium acetate buffer (pH 5.0) and extracted for 2 h at 4 °C with constant mixing. The mixture was centrifuged for 8 min at 3,000 rpm to exclude the SNM. The supernatant containing extracellular enzymes was further filtered through filter paper to remove remaining contaminants.

Table 1
List of analysed *Geosmithia* strains, their ecology, and methods used.

Geosmithia spp.	References	Ecology	Strain	Genbank accession number		Codes used in figures	Extracellular enzymes	Biolog FF	Biolog PM, sporulation	Lipids, ergosterol	Antibiotic activity
				RPB2	*TEF-1α*						
G. sp. 1	Kolařík et al. (2007)	PF, G	CCF4529	submitted	submitted	G1	+	+	+	+	-
G. putterillii	Kolařík et al. (2004)	PF, G	CCF3342	submitted	submitted	Gput	+	+	+	+	+
G. flava	Kolařík et al. (2004)	PF, G	CCF3354	submitted	submitted	Gfla	+	+	+	+	+
G. sp. 8	Kolařík et al. (2008)	PF,	CCF4528	submitted	submitted	G8	+	+	+	+	+
		HWS	CCF4207	submitted	submitted		+	+	+	+	+
G. sp. 9	Jankowiak et Kolařík (2010)	PF, SP	CCF3703	submitted	submitted	G9	+	+	+	+	+
G. sp. 12	Kolařík et al. (2008)	PF, HWS	CCF4274	submitted	submitted	G12	-	+	+	-	-
G. ulmacea	Kolařík et al. (2008)	PF, HWS	CCF4601	submitted	submitted	Gulm	-	+	+	-	+
G. langdonii	Kolařík et al. (2017)	PF, G	CCF3554	HG799926	HG799874.1	Glan	+	+	+	+	-
G. sp. 16	Kolařík et al. (2008)	PF, SP	CCF4201	HE604234.1	HE604206	G16	+	+	+	+	-
G. sp. 20	Kolařík et al. (2007)	PF, G	CCF4527	submitted	submitted	G20	+	+	+	+	+
G. sp. 21	Kolařík et al. (2007)	PF, G	CCF4530	submitted	submitted	G21	+	+	+	+	-
G. sp. 22	Kolařík et al. (2007)	PF, G	CCF3645	submitted	submitted	G22	+	+	-	+	+
G. sp. 24	Kolařík and Jankowiak (2013)	PF, SP	CCF4525	submitted	submitted	G24	+	+	+	+	+
G. sp. 25	Kolařík and Jankowiak (2013)	PF, SP	CCF4205	HE604253	HE604219	G25	+	+	+	+	+
G. sp. 26	Kolařík and Jankowiak (2013)	PF, SP	CCF4223	LN907601.1	LN907596	G26	+	+	+	+	+
G. sp. 27	Kolařík and Jankowiak (2013)	PF, SP	CCF4206	HG799893.1	HG799839.1	G27	+	+	+	+	+
G. sp. 31	Kolařík and Jankowiak (2013)	PF, SP	CCF4526	HE604256	HE604230.1	G31	+	+	+	+	+
G. microcorthyli	Kolařík and Kirkendall (2010)	AF	CCF3861	FM986794	submitted	Gmic	+	+	+	+	+
G. eupagioceri	Kolařík and Kirkendall (2010))	AF	CCF3754	submitted	submitted	Geup	+	+	+	+	+
G. morbida	Kolařík et al. (2011)	HWS, P	CCF3879	submitted	submitted	Gmor	+	+	+	+	+
			1259	submitted	submitted		+	+	+	+	+
			CCF4576	submitted	submitted		+	+	+	+	-
G. rufescens	Kolařík and Kirkendall (2010))	AAF	CCF4524	submitted	submitted	Gruf	+	+	+	+	+
G. sp. CCF4200	unpublished	PF, G	CCF4200	submitted	submitted	G36	+	+	+	+	+
G. cnesini	Kolařík and Kirkendall (2010))	AF	CCF4292	submitted	submitted	Gcne	+	+	+	+	+

Ecology: PF – association with phloem feeding beetles, G – generalist, SF – specialists to *Fagus*, SP – specialist to Pinaceae, HWS – hardwood specialists, P – pathogen, AF –ambrosia fungi, AAF – auxiliary ambrosia fungi, +/- – analysis done/undone.

The spectrum of analysed enzymes is listed in Table 2 together with their function and assays used. We assessed the activities of β-glucosidase, α-glucosidase, cellobiohydrolase, β-xylosidase, N-acetylglucosaminidase, arylsulfatase, phosphomonoesterase, phosphodiesterase, alanine- and leucine aminopeptidases with fluorogenic substrates 4-methylumbelliferone (MUF) and 7-amido-4-methylcoumarin (AMC) (Megazyme, Ireland); individual fluorogenic substrates are stated in Table S1. Fluorescence of the released reaction products was measured as described previously (Baldrian, 2009) using a method modified from Vepsäläinen et al. (2001). The fluorescence value of each MUF/AMC fluorogenic substrate was corrected by subtraction of background fluorescence of the SNM medium.

Table 2
List of analyzed enzymes, their function and assays.

Process	Enzyme	Assay
Cellulose degradation	β-glucosidase	MUF
	cellobiohydrolase	MUF
	endoglucanase	depolymerization
Degradation of polysaccharides	α-glucosidase	MUF
	N-acetylglucosaminidase	MUF
Degradation of hemicelluloses	β-xylosidase	MUF
	endoxylanase	depolymerization
S acquisition	arylsulfatase	MUF
P acquisition	phosphomonoesterase	MUF
	phosphodiesterase	MUF
N acquisition	alanine aminopeptidase	AMC
	leucine aminopeptidase	AMC
Lignin transformation	peroxidases	DMAB
	laccase	ABTS
	oxidases	DMAB

MUF – methylumbelliferone, AMC – amidomethylcoumarin, DMAB – 3,3-dimethylaminobenzoic acid, ABTS – 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid.

Laccase (EC 1.10.3.2) activity was measured by monitoring the oxidation of ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (Bourbonnais and Paice, 1990);) in citrate-phosphate buffer (100 mM citrate, 200 mM phosphate, pH 5.0). The formation of the resulting green dye was evaluated spectrophotometrically at 420 nm. The activities of manganese peroxidase (EC 1.11.1.13; MnP), Mn-independent peroxidases (MIP) and oxidases were assayed according to Ngo and Lenhoff (1980) in succinate-lactate buffer (100 mM, pH 4.5). MBTH (3-methyl-2-benzothiazolinone hydrazone) and DMAB (3,3-dimethylaminobenzoic acid) are oxidatively coupled by the action of the enzyme, and the formation of a purple indamine dye product was detected spectrophotometrically at 595 nm. The activities of MIP were measured in samples without manganese sulfate, which was substituted by an equimolar amount

of ethylenediaminetetraacetate. For the detection of the activity of oxidases, hydrogen peroxide was substituted by water. Activities of endo-1,4-b-glucanase (EC 3.2.1.4) and endo-1,4-b-xylanase (EC 3.2.1.8) were measured with Azo-CM Cellulose and Azo-Xylan, respectively, using the protocol and calibration curves of the supplier (Megazyme, Ireland).

2.3.2. Biolog analysis

Biolog FF MicroPlate™ (FF) and Biolog Phenotype Micro-Arrays™ (PM) (Biolog, Inc., Hayward, CA) were used to evaluate the assimilation profiles of carbon (FF), nitrogen (PM3B), phosphorus, sulfur (PM4A) and nutrient supplement (PM5) sources following manufacturer's instructions. The inoculated plates were then incubated in the dark at 25 °C and absorbance at 750 nm was used to measure mycelial growth at 24, 48, 72, 96 and 168 h. Two technical replicates per strain were prepared for FF plates and one replicate for PM plates. An absorbance reading taken 96 h after the inoculation was included in the analysis. The absorbance of the negative control was subtracted from all substrates within one plate and negative values were assigned a value of zero (Garland, 1996).

In addition, substrates in FF plates were divided into several guilds (carbohydrates, carboxylic acids, amino acids, amines/amides, polymers and miscellaneous) following Dobranic and Zak (1999), with the exceptions of the amino acids guild, which was combined with the guild of amines/amides, and the guild of polymers, which was transferred to the 'miscellaneous' guild containing various type of substrates. The reason for the merging was the low number of substrates within the particular guilds. We also evaluated sporulation in each PM well using a stereomicroscope 1 week after inoculation. Sporulation was classified into four levels: 0 – none, 1 – low, 2 – middle, 3 – high.

2.3.3. Analysis of total fatty acids and ergosterol

For measurements of fatty acids and ergosterol content, fungi were grown in a Petri dish for 11 d on MEA overlaid with a cellophane disc to physically separate the fungal biomass from the medium. Total fatty acids from lyophilized fungal biomass were extracted in a mixture of chloroform-methanol-phosphate buffer (1:2:0.8). Fatty acid fractions were separated using solid-phase extraction cartridges (LiChrolut Si 60, Merck), and the samples were subjected to mild methanolysis (Šnajdr et al., 2008). The free methyl esters of fatty acids were analyzed by gas chromatography-mass spectrometry (Varian 3400; ITS-40, Finnigan). The gas chromatography (GC) instrument was equipped with a split/splitless injector, and a DB-5MS column was used for separation (60 m, 0.25 mm i.d., 0.25 μm film thickness). The temperature programme started at 60 °C and was held for 1 min in splitless mode. Then, the splitter was opened and the oven was heated to 160 °C at a rate of 25 °C/min. The second temperature ramp was up to 280 °C at a rate of 2.5 °C/min; this temperature was maintained for 10 min. The solvent delay time was set to 8 min. The transfer line temperature was set to 280 °C. Mass spectra were recorded at 1 scan/s under electron impact at 70 eV, with a mass range of 50–350 amu. Methylated fatty acids were identified according to their mass spectra using a mixture of chemical standards obtained from Sigma.

The content of ergosterol in dry weight of 11 d old mycelium was analyzed by the method developed by Bååth (2001) and modified by Šnajdr et al. (2008). Lyophilized samples were sonicated (90 min, 70 °C) in 1 ml of cyclohexane and 3 ml of 10% KOH in methanol. Then, 1 ml of distilled water was added to samples and ergosterol was triple-extracted with 2 ml of cyclohexane. The samples were dried under nitrogen and dissolved in 1 ml of methanol by heating to 40 °C for 15 min. The samples were analyzed isocratically using a Waters Alliance high-performance liquid chromatography (HPLC) system (Waters, USA) with methanol as a mobile phase at a flow rate of 1 ml/min. Ergosterol was detected using a UV light at 282 nm.

2.3.4. Antibiotic production assessment

To quantify the diversity of antibiotic compounds produced by individual *Geosmithia* species, we used the Kirby-Bauer disk diffusion susceptibility approach. An optimized extraction procedure was developed by testing different solvents and sorbents. Subsequent ultra-high-performance liquid chromatography method with diode array detection (UPLC-DAD) analysis was applied to cover the broadest spectrum of extracted extracellular compounds (Tylova et al., 2011). After 14 d of submerged cultivation of *Geosmithia* spp., the fermentation broth (MEA without agar, shaken on a rotary shaker, 3.4 Hz) was centrifuged (15 min at 4,000 g) and then filtered through a 2-μm glass microfibre filter (Whatman, UK). Prior to solid phase extraction (SPE), the pH of the samples was adjusted to 3 with formic acid (98–100%). The SPE was performed using 60-mg Oasis MCX cartridges. The sorbent was conditioned with 2 ml of methanol followed by equilibration with 2 ml of Milli-Q water; 50 ml of fermentation broth from each strain was passed through the cartridge at the flow rate of 3 ml/min, and the sorbent was then rinsed with 2 ml of water and 2 ml of a formic acid/water solution (1:99, v/v). Afterwards, the cartridge was air-dried and the secondary metabolites were eluted using 2 ml of methanol. The methanol extracts were evaporated to dryness and reconstituted in 100 μl of methanol. The organisms used for antimicrobial activity testing included Gram-positive bacteria *Kocuria rhizophila* CCM = ATCC9341, previously known as *Micrococcus luteus*, Gram-negative bacteria *Escherichia coli* ATCC3988, yeast *Saccharomyces cerevisiae* CCM8191 (=ATCC9763) and the following filamentous fungi: saprophyte *Penicillinum decumbens* CCF4423, insect pathogenic *Beauveria bassiana* CCF4422 and endophyte *Graphium fimbriisporum* CCF4421. Bacteria were cultivated on a beef extract medium containing: beef extract 10 g/l, peptone 10 g/l, NaCl 5 g/l and agar 20 g/l; pH 7.2 adjusted by NaOH. Yeasts were cultivated on a yeast extract medium consisting of: glucose 40 g/l, peptone 4 g/l, yeast extract 5 g/l and agar 20 g/l; pH 7.0 adjusted by NaOH. Filamentous fungi were cultivated on MEA. The plates were then overlaid with the indication organism (suspended in a sterile saline solution and vortexed until a smooth suspension was obtained). The filter paper discs were impregnated with 25 μl of fungal extract and air-dried. A disc impregnated with methanol served as a blank control. Afterwards, the impregnated discs were placed on to the surface of the agar. Growth inhibition zones, indicating the antimicrobial activity of the fungal extract, were detected after 24 h of incubation at 30 °C for bacteria, after 24 h at 38 °C for yeast and after 48 h at 24 °C for filamentous fungi. Following the incubation, inhibition zones were evaluated and measured (Bauer et al., 1966).

2.3.5. Statistical analyses

Our objective was to determine the degree to which functional traits of *Geosmithia* are driven by their ecology and constrained by their evolutionary histories. This was accomplished by regression of phylogenetic eigenvectors and ecological axes against several functional trait datasets in a variation partitioning analysis (Fig. 1).

Predictor variables 1: phylogenetic eigenvectors – Phylogenetic eigenvectors are vector representations of phylogenetic relationships among taxa and can be used as covariates in linear and non-linear models to account for variation among taxa (e.g. ecological, morphological, physiological, etc.) that is attributed to phylogenetic relationships (Diniz-Filho et al., 2012). Phylogenetic eigenvectors are orthogonal and together describe all scales of phylogenetic variation among taxa in the dataset. They can, therefore, be used to

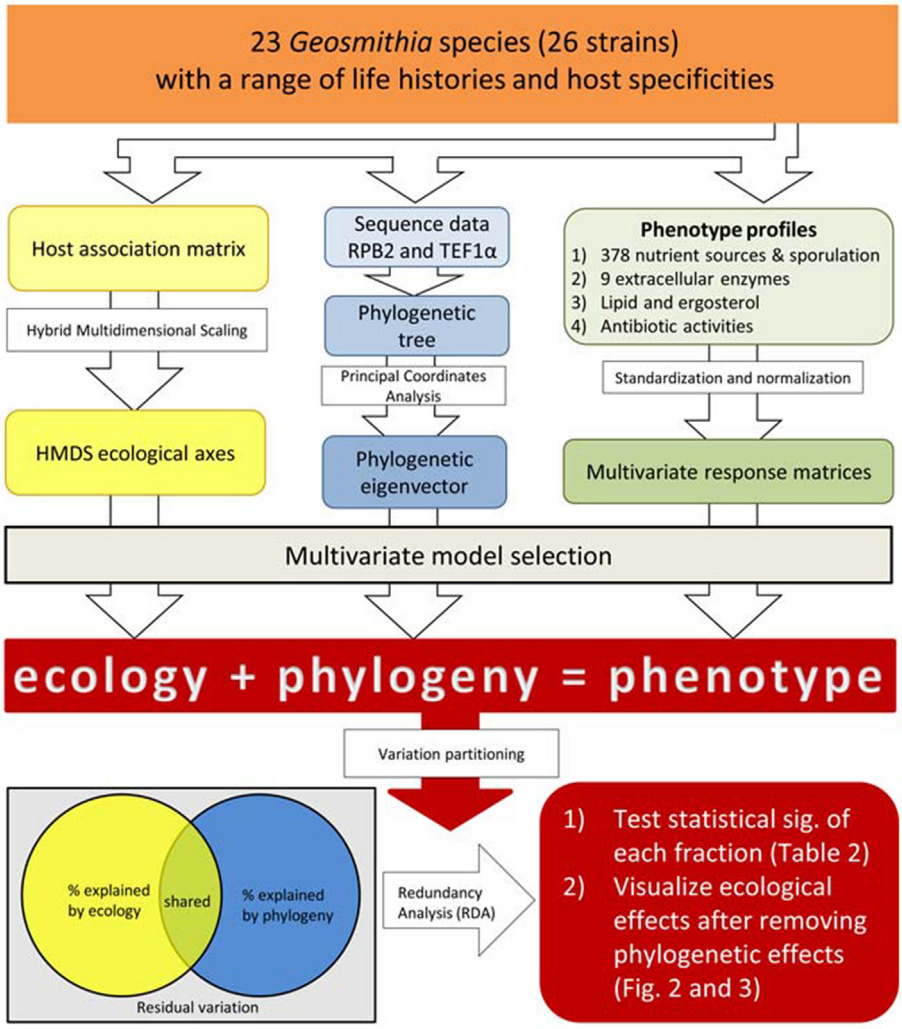

Fig. 1. Analytical pipeline used to separate the effects of ecology from those of phylogenetic history along several axes of phenotypic variation in *Geosmithia* fungi. Our pipeline provides: (1) a quantitative assessment of phenotypic variation explained by ecological and phylogenetic differences among fungi, (2) a statistical test of the significance of each source of variation, while accounting for variation explained by the other source, and (3) visualizations of the independent effects of ecology on phenotypic variation after removing phylogenetic effects.

rigorously account for broad- and fine-scale phylogenetic correlations among taxa (Tedersoo et al., 2013). To generate phylogenetic eigenvectors for *Geosmithia*, branch lengths from the best scoring tree obtained by the phylogenetic analysis were imported using the 'ape' R package (Paradis et al., 2004); (R Developement Core Team, 2010) and converted into an ultrametric phylogram, which is an additive phylogenetic tree for which all paths from the root to branch tis are equal. From the ultrametric phylogram, a pairwise distance matrix of branch lengths was then calculated for all taxa. Principal coordinates analysis was then used to calculate phylogenetic eigenvectors from the pairwise distance matrix of branch lengths (Fig. 2, Diniz-Filho et al., 2012).

Predictor variables 2: ecological axes: The contribution of realized niches to functional traits was tested by three different approaches:

1. **Ecological affinities** among *Geosmithia* species were calculated from their known ranges of associations with host plant families (Fig. 3). A binary matrix of observed associations between *Geosmithia* species and plant families was imported using the 'vegan' R package (R Developement Core Team, 2010; Oksanen et al., 2013) and used to calculate a Jaccard distance matrix. The Jaccard distances showed signs of distance saturation (52% of distances were equal to 1) because many *Geosmithia* species shared no observed host taxa. To examine ecological relationships among taxa with no shared host species, hybrid multidimensional scaling (HDMS) was used to ordinate all *Geosmithia* species according Jaccard distance, using the monoMDS() function and allowing equal dissimilarities to have different fitted values (using the 'hybrid' method and 'weak treatment' for ties; see Tuomisto et al., 2012) and supporting information for the monoMDS() function in the 'vegan' package in R 3.3.3). The dimensionality ($k = 3$) was chosen based on an assessment of a scree plot (McCune et al., 2002). The resultant HDMS axes scores were used in subsequent variation partitioning to represent ecological relationships among *Geosmithia* species. Simulation studies have established HDMS as an effective means to resolve relationships among entities with no shared qualities for subsequent variation partitioning analyses (Tuomisto et al., 2012).
2. To assess effects of generalist versus specialist ecologies (TAXA), independent of plant host composition, we also included the **number of plant families** each species is known to associate with.

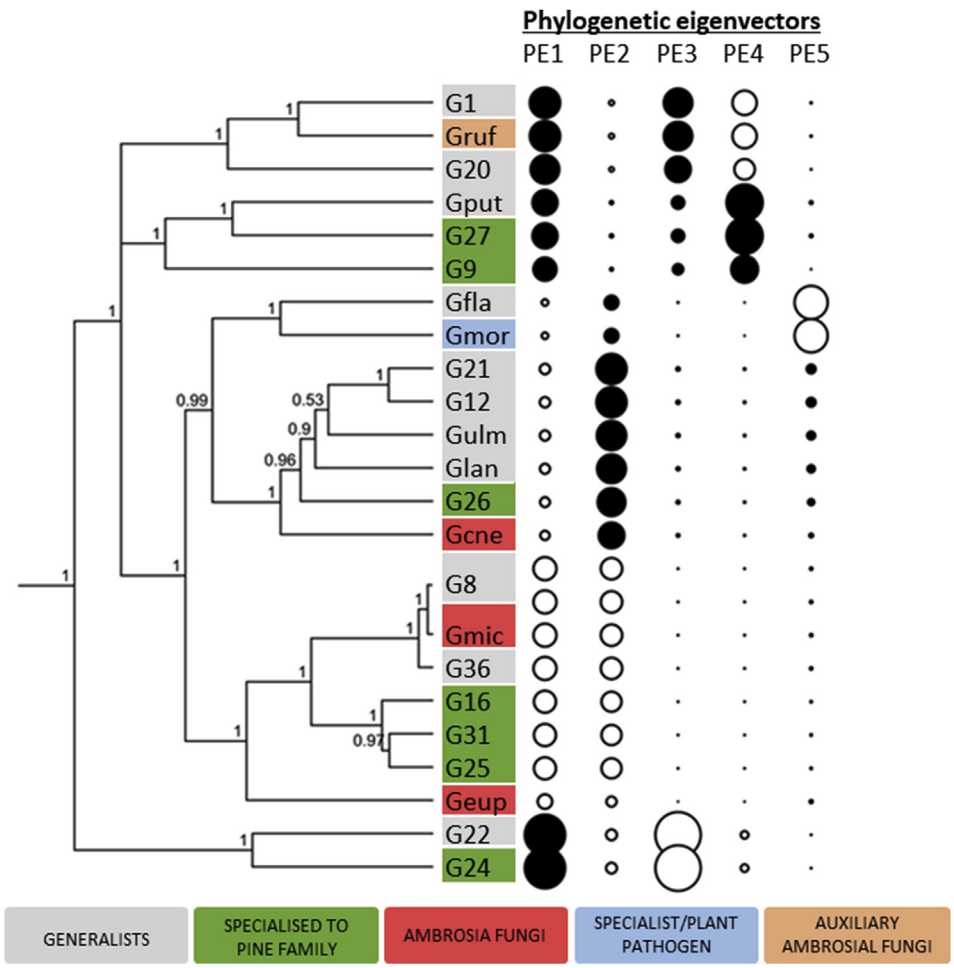

Fig. 2. Bayesian inference tree and graphical representation of phylogenetic eigenvectors used in variation partitioning analysis. Circles represent eigenvector coordinates. The size of each circle is scaled to absolute values, and black-and-white circles represent negative and positive values, respectively.

3. To determine the effects of insect fungiculture (AMB), we also included a binary variable differentiating between **ambrosial** or non-ambrosial fungi.

We used a Mantel test (mantel() function in the 'vegan' R package) to test for correlations between the two predictor dataset, namely the phylogenetic and ecological distance matrices.

Response variables and variation partitioning: A response matrix was constructed for each of several functional trait datasets. Because of high variability in the magnitude of measurements for elements within matrices, all columns of each functional trait matrix were standardized by the column maximum and normalized as needed. To avoid over-fitting the subsequent variation partitioning model, we used forward model selection of phylogenetic eigenvectors and, separately, forward selection of ecological HDMS based on adjusted R-squared and an alpha significance level determined by 9,999 permutations (Blanchet et al., 2008). We also only included phylogenetic eigenvectors that captured more than two percent of the variation among isolates. Model selection was carried out using the 'packfor' R package (Blanchet et al., 2008) with a significance level of $\alpha = 0.1$ to identify potentially important explanatory vectors, as in Tedersoo et al. (2013). Only the single best predictor was retained when automated selection did not retain any predictors. Variation partitioning was then used to determine the amount of variation explained by the selected ecological and phylogenetic models, and the amount of explained variation that was shared between explanatory variables. Redundancy analysis (RDA) was used to test the statistical significance of testable components of the variation partitioning, namely the effect of ecology with phylogenetic effects removed and the effect of phylogeny with ecological effects removed (Peres-Neto et al., 2006). For each functional trait dataset with a significant ecological model, we visualized the RDA model that represented the variation of the given functional trait among *Geosmithia* species that was explained by their ecological distance (approximated by host plant associations), with phylogenetic effects removed.

We also investigated relationships between ecological and phylogenetic predictors with the univariate response of the diversity of extracellular enzymes produced by each species and the diversity of substrates that each species could utilize. Biolog™ growth profiles were separated for carbon, nitrogen, phosphorus and nutrient supplement assays prior to univariate analyses. For each response variable, diversity was calculated as Simpson's index of diversity. Simpson's diversity was transformed to real number equivalents (Jost, 2006), which reflects the richness and evenness of growth profiles by approximating the number of substrates with an equal measured growth rate for each species. Model selection was used as described above to select significant ecological and phylogenetic predictors. Final models were fit using the lm() function in the base R package.

Differences in the amounts of ergosterol, carbon guilds and saturation of fatty acids between ecological groups were evaluated

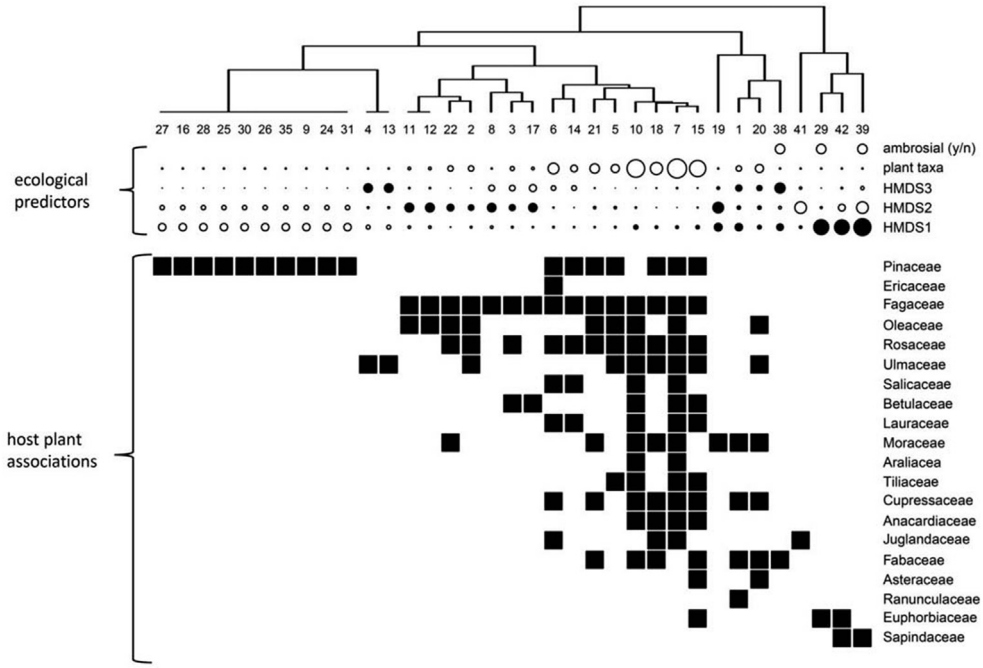

Fig. 3. Ecological variables used in variation partitioning. At the top, a hierarchical clustering dendrogram shows the ecological relationships among *Geosmithia* species (with the numbers of species as labels). The circle plot illustrates ecological variables, including hybrid multidimensional scaling (HMDS) axes, the number of known host plant families and the presence of an ambrosial lifestyle. Circle size is scaled to the absolute value for each variable. White indicates positive values and black negative values. HMDS scores were obtained by ordination of a Jaccard distance matrix calculated from the binary host plant association matrix shown at the bottom of the figure. The HMDS axes describe the dimensions of variation among plant associations of *Geosmithia* species. For instance, HMDS1 captures the difference between pine specialist taxa (cluster on the far left), generalists (middle) and ambrosia fungi (cluster on the far right). Used code for species: 13 – *G. ulmacea*, 6 – *G. putterillii*, 10 – *G. omnicola*, 18 – *G. lavendula*, 38 – *G. microcorthyli*, 41 – *G. morbida*, 29 – *G. cnesini*, 42 – *G. rufescens*, 39 – *G. eupagioceri*.

using Kruskal-Wallis statistics supplemented with a Mann-Whitney pairwise comparison and Bonferroni correction. Both analyses were carried out in PAST software (Hammer et al., 2001).

3. Results

We identified the independent impacts of ecology and phylogeny on functional traits in *Geosmithia*, which allowed a robust assessment of their individual effects. This result is supported by the lack of a correlation between phylogenetic and ecological distances according to Mantel's test (Fig. S1), and was confirmed by variation partitioning analyses, which showed no significant shared fraction between ecological and phylogenetic predictors in any of the functional trait datasets. Overall, ecology was a much better predictor of the phenotype in every analysis (Table 3). In cases where model selection retained some phylogenetic eigenvectors (Table 4), the phylogenetic fraction was not significant in variation partitioning (Table 3), except lipid analysis where the phylogenetic fraction was small but statistically significant.

3.1. Enzymatic analysis

The ecological variables explained more than 20% of the variation observed in the enzymatic composition (Table 3) which was well predicted by ecological affinities (HMDS predictors) (Table 5). The initial model selection also retained the phylogenetic eigenvector PE4 (Table 4). However, PE4 did not explain a significant fraction in the variation partitioning analysis (Table 3). In a univariate analysis, there were no significant relationships between ecology or phylogeny and enzyme diversity. This indicates that all clades and ecological groups of *Geosmithia* had similar diversity in their enzyme suites, but the identity of the enzymes in those suites varied depending on the ecology of *Geosmithia* species. *Geosmithia* pine specialists formed a distinct group in the RDA analysis (Fig. 4), which may reflect their ecological specialization to the pine environment. They were associated with higher production of lignin and cellulose-transforming enzymes (laccase, oxidase, manganese peroxidase, β-glucosidase and cellobiohydrolase), phosphodiesterase and leucine aminopeptidase. Generalists were characterized by the production of enzymes cleaving hemicellulose (β-xylosidase, α-glucosidase and endoxylanase) and arylsulfatase, and by almost absence of enzymes modifying lignin. The pathogen *G. morbida* was unique in production of all enzymes cleaving the structural components of the plant cell wall (i.e. cellulose, hemicellulose and lignin); nevertheless, their production was moderate. Ambrosia fungi were dissimilar to each other in the composition of their extracellular enzymes. *G. microcorthyli* exhibited the highest activities of lignin transforming enzymes (laccase, Mn-peroxidase and oxidases), *G. eupagioceri* displayed high activity of endoxylanase. *G cnesini* and the auxiliary ambrosial *G. rufescens* did not exhibit any enzymes linked with the degradation of lignocellulose. The ability to degrade chitin (N-acetylglucosaminisase) was detected in all studied strains and was not linked with species ecology (Table S1).

3.2. Biolog MicroPlates analysis

Ecological predictors explained 17% of the compositional variation in growth performance on Biolog™ substrates whereas phylogenetic relationships were not significant predictors (Table 3). The number of plant taxa (TAXA) was the strongest ecological predictor, though one dimension of ecological affinities (HMDS2) and the variable differentiating between ambrosial and free-living (AMB) fungi were also significant (Table 5, Fig. 5).

Table 3
Proportion of variation in each functional trait dataset explained exclusively by ecology (left shaded column), exclusively by phylogeny (right shaded column) and the shared portion (centre shaded column). Bold font indicates statistical significance according to partial RDA (*p < 0.05, **p < 0.01). The shared portion of variation explained by both ecology and phylogeny cannot be tested for significance.

Functional trait datasets	ecology	ecology + phylogeny	phylogeny
Extracellular enzymes	**0.204****	0.016	0.014
Biolog™ - growth	**0.170****	0.020	0.01
Biolog ™ - sporulation	**0.122***	0.005	0.035
Lipids	**0.060***	0.000	**0.044***

Table 4
Selected phylogenetic vectors from forward model selection.

Functional traits	phy. eigenvectors	R-squared	cum. adj R^2	p-value
Extracellular enzymes	PE4	0.081	0.030	0.098
Biolog™ - growth	PE2	0.074	0.025	0.064
Biolog™-sporulation	PE2	0.089	0.041	0.074
Lipids	PE1	0.898	0.039	0.064

Table 5
Selected ecological vectors from forward model selection for multivariate phenotype data. HMDS axes are visualized in Fig. 3. TAXA = number of plant families known to associate with, AMB = ambrosial life history.

Functional traits	predictors	R-squared	cum. adj R^2	p-value
Extracellular enzymes	HMDS2	0.116	0.066	0.006
	HMDS1	0.106	0.130	0.008
	HMDS3	0.095	0.188	0.026
	AMB	0.068	0.220	0.076
Biolog™ - growth	TAXA	0.173	0.129	0.001
	HMDS2	0.087	0.178	0.043
	AMB	0.100	0.245	0.020
Biolog™ - sporulation	AMB	0.132	0.086	0.048
	HMDS2	0.082	0.127	0.075
Lipids	HMDS1	0.101	0.051	0.037

Ecology also explained variation in the total diversity of substrate utilization by each *Geosmithia* species (Table 6). Diversity in utilized carbon and nitrogen sources was best explained by predictors of ecological affinity (HMDS1 and HMDS2). By contrast, the best predictor of diversity in the utilization of phosphorus and sulfur substrates and nutrient supplement was the number of known plant hosts (TAXA) and ambrosial status, respectively (Table 6). Pine specialists and the pathogen *G. morbida* have lost a number of metabolic pathways. This corresponds to the significant difference in substrate diversity. The proportional representation of carbon guilds remained similar between the ecological groups (Kruskal Wallis, p > 0.4) whereas specialists and the plant pathogen exhibited a slight shift towards saccharides. Ambrosial life history (AMB) and ecological affinities (HMDS2) were the best predictors explaining variation in sporulation on Biolog™ microplates (Table 5). The sporulation of ambrosia fungi and the auxiliary ambrosia species *G. rufescens* was supported on nutrient supplement substrates whereas that of hardwood specialists *G.* sp. 12, *G. ulmacea* and *G.* sp. 8 was enhanced on phosphorus and sulfur sources (Fig. 6).

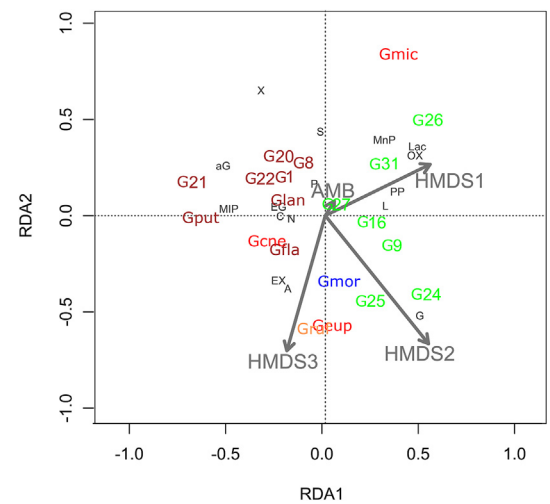

Fig. 4. Partial RDA showing variation among *Geosmithia* species in extracellular enzyme profiles that is explained by ecology. Arrows indicate retained significant ecological predictor variables – ecological affinities (HMDS axis) and ambrosial life history (AMB). Green – pine specialists, brown – generalists, red – ambrosial species, blue – pathogen, orange – auxiliary ambrosia species. Species codes are listed in Table 1. Abbreviation of enzymes: A – alanine aminopeptidase, αG – α-glucosidase, C – cellobiohydrolase, EG – endoglucanase, EX – endoxylanasa, G – β-glucosidase, L – leucine aminopeptidase, Lac – laccase, MIP – Mn-independent peroxidases, MnP – Mn-peroxidases, N – N-acetylglucosaminidase, Ox – oxidases, P – phosphomonoesterase, PP – phosphodiesterase, S – arylsulfatase, X – xylosidase.

3.3. Ergosterol and total fatty acids

The proportional content of ergosterol in dry weight ranged from 0.23 to 0.81%, with a mean value of 0.42%. Twenty-four different fatty acids were detected in *Geosmithia* (Table S2). The most abundant fatty acids were palmitic acid (16:0), linoleic acid (18:2ω6,9), oleic acid (18:1ω9), stearic acid (18:0) and an unidentified acid (20:4). Ecological affinity (HMDS1) was the best predictor, although phylogeny also significantly contributed to the lipid variation. All three ambrosial species were characterized by high proportions of oleic acid. Nevertheless, *G.* sp. 8 and *G.* sp. CCF4200, sister species to the ambrosial *G. microcorthyli*, displayed the same pattern, so the ambrosial vs. free-living predictor was not significant (Table 5, Fig. 7). The proportional lipid content in dry weight was similar among the *Geosmithia* species, with a median of approximately 10%. We divided fatty acids into three groups: saturated, unsaturated (with one double bond) and

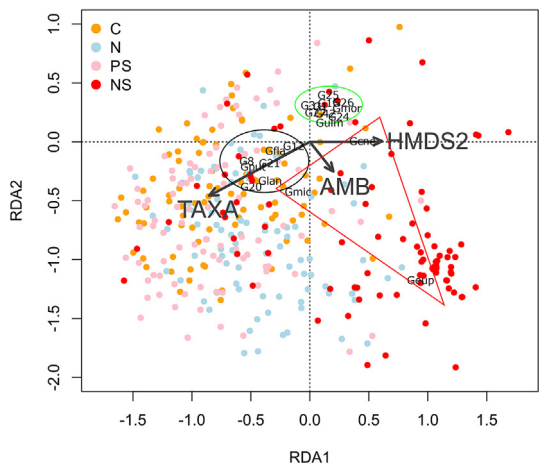

Fig. 5. RDA showing variation among *Geosmithia* species in Biolog™ growth profiles that is explained by ecology. Arrows indicate the retained significant ecological predictor variables – ecological affinities (HMDS2), number of plant taxa (TAXA) and ambrosial life history (AMB). Coloured circles represent scores for each Biolog substrate. *Geosmithia* generalists (black ellipse) use a greater number of carbon (orange), nitrogen (light blue), and phosphorus and sulfur (pink) sources than pine specialists (green ellipse). Nutrient supplements (red symbols) were associated with higher growth of ambrosial species (red triangle). Species codes are listed in Table 1.

polyunsaturated. In congruence with previous results (HMDS1 vector), pine specialists were statistically separated from ambrosia fungi in all fatty acid groups (Kruskal-Wallis, $p < 0.05$). Ambrosia fungi had the highest proportion of saturated and unsaturated fatty acids but contained the lowest proportion of polyunsaturated fatty acids. The pathogen *G. morbida* displayed a shift from unsaturated to polyunsaturated fatty acids, which constituted up to nearly 40% of the total fatty acids.

3.4. Kirby-Bauer disk diffusion susceptibility test

We detected antibiotic production against at least one model organism in nearly half of the strains tested, but it was not connected with species ecology. Inhibition of the insect pathogen *Beauveria bassiana* and Gram-positive bacteria *Kucuria rhizophila* was the most frequent (Table S3). The most potent species were pine specialist *G.* sp. 9, which inhibited all model organisms, followed by the ambrosia species *G. microcorthyli* and the pathogen *G. morbida*, which inhibited five of six model organisms. Nevertheless, high intra-species variability was documented in some species, which indicates either that this trait is strain-specific or that our method was not sufficiently sensitive.

4. Discussion

The aim of our study was to attribute functional trait variation among *Geosmithia* species to ecological and phylogenetic effects. This enabled us to identify traits that are under selective pressure, and perhaps related to the formation of particular symbiotic associations. All the features under study, except ergosterol content and the production of antibiotics, were better explained by the ecology of each *Geosmithia* species than by their phylogenetic relationships. These results suggest that *Geosmithia* phenotypes are highly adaptable and not heavily constrained by phylogenetic history.

Nevertheless, we also found some traits common for all *Geosmithia* species. Their growth was supported on urea, uric acid and partly ammonia on Biolog™ plates which demonstrates their ability to recycle nitrogen, possibly from beetle excretions. They also possess an exo-enzyme cleaving chitin, the main component of the fungal cell wall and of the beetle exoskeleton. As the concentration of nitrogen in beetle galleries markedly affects beetle fitness (Ayres et al., 2000), nitrogen recycling seems to be of great importance. We also detected antibiotic activities in the majority of *Geosmithia* species, which is in concordance with spectrum of secondary metabolites detected earlier (Stodůlková et al., 2009, 2010). *Geosmithia* fungi are thereby able to actively affect populations of other fungi and/or bacteria in beetle galleries with which they compete for growth substrate, nutrients uptake and beetle transfer. Notable allelopathy observed in ambrosial *G. microcorthyli* suggests that, in cooperation with fungus-cultivating beetles (Batra, 1966; Beaver, 1989), ambrosia fungi themselves could participate in suppression of airborne fungal contaminants occurring in young ambrosial galleries. Even though some fungal mycoparasites act as promising antagonists against the phytopathogenic *G. morbida* (Gazis et al., 2018), this species possesses strong antibiotic capabilities. This might explain its omnipresence in the walnut twig beetle ecosystem. The total ergosterol and lipid content of *Geosmithia* species was not explained by species ecology. We concur with Bentz and Six (2006) that these features are not under selective pressure in the fungus-beetle association.

We found that metabolic capabilities, extracellular production and spectrum of fatty acids are driven by species ecology with only little effect of phylogenetic relationships. Breadth of the niche (phylogenetic diversity of plant hosts) was the best predictor tested, which supports our hypothesis. *Geosmithia* generalists, with the most unrestricted relationships with beetles and host trees are enzymatically versatile which suggest absence of any specific co-evolution with the vector beetles. This is reflected in their ability to grow on a broad spectrum of substrates (Biolog™ analysis), in which they are similar to fungal saprotrophs such as the ubiquitous *Trichoderma/Hypocrea* species (Kubicek et al., 2003). *Geosmithia* generalists tend to cleave hemicellulose by high endoxylanase and xylosidase activities and by extensive growth on arabinose and galactose, the most abundant saccharides in hemicellulose chains (Cosgrove, 1997; Santiago et al., 2013). They massively sporulate within 1 week on a broad spectrum of carbon and nitrogen

Table 6
Best linear models for total diversity of carbon, nitrogen, phosphorus, and nutrient supplement substrates that each species could assimilate. No phylogenetic eigenvectors were retained for any substrate type. HMDS axes = plant affinities are visualized in Fig. 3, TAXA = number of plant families, AMB = ambrosial life history. For the best carbon model, adjusted $R^2 = 0.552$, $F_{2,18} = 13.34$, $p < 0.001$. For the best nitrogen model, adjusted $R^2 = 0.448$, $F_{2,18} = 9.12$, $p = 0.002$. For phosphorus, adjusted $R^2 = 0.368$, $F_{2,19} = 9.274$, $p = 0.007$. For nutrient supplements adjusted $R^2 = 0.448$, $F_{2,19} = 12.650$, $p = 0.002$.

Models	Predictor	Estimate	Standard error	t	p-value
Carbon	HMDS2	−13.584	2.968	−4.577	<0.001
	HMDS1	−6.207	2.000	−3.104	0.006
Nitrogen	HMDS1	−9.893	2.813	−3.517	0.002
	HMDS2	−12.373	4.176	−2.963	0.008
Phosphorus and sulphur	TAXA	3.317	1.030	3.045	0.007
Nutrient supplement	AMB	21.840	6.142	3.556	0.002

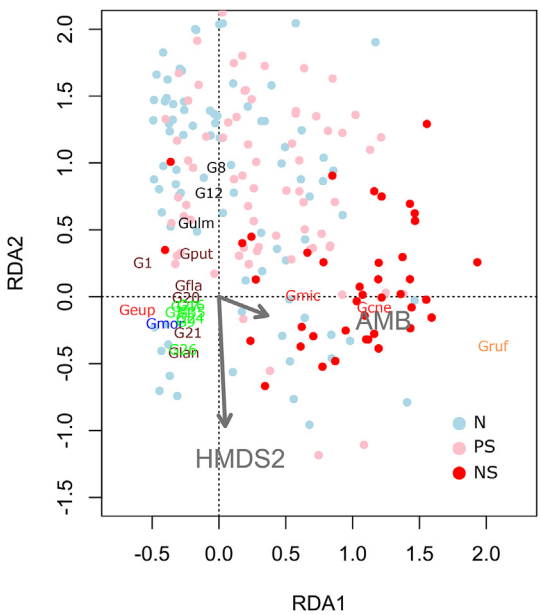

Fig. 6. Partial RDA showing variation among *Geosmithia* species in Biolog™ sporulation profiles that is explained by ecology. Arrows indicate the retained significant ecological predictor variables – ecological affinities (HMDS2) and ambrosial life history (AMB). Coloured circles represent scores for each Biolog substrate. N – nitrogen sources, PS – phosphorus and sulfur sources, NS – nutrient supplementary sources. Generalist are represented by brown color, pine specialists by green, pathogen by blue, ambrosial species by red, auxiliary ambrosial species by orange and hardwood specialists by black. Species codes are listed in Table 1.

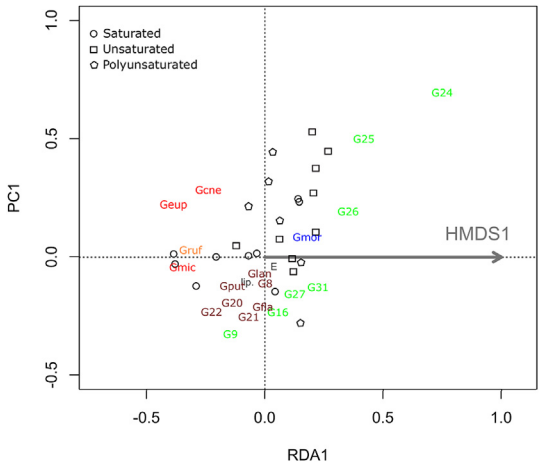

Fig. 7. Partial RDA showing variation among *Geosmithia* species in lipid (lip) and ergosterol (E) profiles that is explained by ecology. The arrow indicates the retained significant ecological predictor – ecological affinities (HMDS1). Generalist are represented by brown colour, pine specialists by green, pathogen by blue, ambrosial species by red and auxiliary ambrosial species by orange. Values of fatty acids are divided by their saturation. Species codes are listed in Table 1.

substrates which reflects their ability to rapidly grow and sporulate in beetle galleries. According to the *r-K* selection theory, species respond to ecological trade-offs by investing either into large amounts of less developed offspring, or into production of fewer but competitively stronger offspring. This theory has been also applied to microorganisms (Andrews and Harris, 1986), where species belonging to *r*-strategists are characterized by quick growth on nutrient rich substrates under optimal conditions and frequent occurrence of disturbances. In contrast, *K*-strategists grow well in stable and nutrient poor environments (they are specialized). *Geosmithia* generalists live in association with a broad range of bark beetles species infesting multiple plant families. Consequently, they have to cope with unpredictable substrate switches, which could have a similar effects as disturbances. *Geosmithia* generalists can be characterized by quick growth and early and massive production of small conidia. For these reasons, similarly to Veselská and Kolařík (2015), we consider *Geosmithia* generalist *r*-strategists.

With the exception of increased sporulation on sulphur sources, angiosperm tree specialists (*G.* sp. 12, *G. ulmacea* and *G.* sp. 8) were similar to generalists in their enzymatic potential. By contrast, *Geosmithia* pine specialists expressed strong narrowing of their enzymatic richness by growing only on a small number of Biolog™ substrates. We propose that the limitation of *Geosmithia* specialists to a single plant family *Pinaceae* and possible long-term co-evolution with their vectors (Kolařík and Jankowiak, 2013) has led to a loss of unnecessary metabolic pathways, a trend documented from a variety of specialists (Kelley and Farrell, 1998). Obligatory mutualisms between phloem-feeding bark beetles and fungi are mostly known from beetles specialized on conifers. This might indicate a tendency of conifer bark beetles to gain necessary nutrients through mycophagy (Six, 2013). *Geosmithia* pine specialists were also unique in their extracellular enzymatic profiles. Their enzyme suite, including cellobiohydrolase, β-glucosidase, laccase and manganese peroxidase, suggests the capacity to alter the structural components of plant tissues, i.e. cellulose and lignin, suggesting the capacity to fully utilize their plant substrate. They are also characterized by weaker sporulation compared to *Geosmithia* generalists and by a tendency to create large conidia with increased DNA content (Veselská and Kolařík, 2015). Larger conidia accumulate more nutrients than smaller ones, which enhances their resilience to negative climatic factors during germination (Kauserud et al., 2011) and makes them a better diet source for beetles. We suppose that substrate and vector specificity to the pine environment and the long-term co-evolution between these beetles and *Geosmithia* led to a decrease in the total fungal diversity in beetle galleries and thus to lower competition between fungal species for transport on beetle bodies. *Geosmithia* pine specialists thus take advantage of competitive release and resulting niche stabilization. As a consequence, they appear to invest in progeny fitness by conserving nutrient sources and energy by enzymatic streamlining and by the production of large conidia with greater resilience. This classifies them among *K*-strategists.

The entire genus *Geosmithia* displays high enzymatic versatility which is especially manifested in *G. morbida.* Although the genome of *G. morbida* is not enriched in CAZymes compared to non-pathogenic *Geosmithia* species (Schuelke et al., 2017), we have identified that this single species is able to modify, to a certain extent, all structural components of the plant body (lignocellulose and hemicellulose – Table S1). We, therefore, propose that the enzymatic versatility of *G. morbida* is one of its virulence factors. *G. morbida* is a weak pathogen in its native region and became a threat only after it spread outside this area. It is possible that this enzymatic versatility, accompanied by the aggressive feeding behaviour of the twig beetle *Pityophthorus juglandis* and environmental pressure on its host in the expanded geographic range (Tisserat et al., 2009; Hulcr and Dunn, 2011), are reasons for the occasional *Juglans nigra* dieback.

Adaptive change in enzymatic equipment shaped by mutualistic symbiosis has been described in fungal symbionts of ants (De Fine Licht et al., 2010). However, the specific nutritional ecology of ambrosia fungi has rarely been studied. Similarly to Huang et al. (2018), we found that the ambrosial *Geosmithia* are not highly enzymatically specialized. In general, ambrosia fungi are obligatorily dependent on transmission by specific ambrosia beetle species; however, they are often generalists with respect to the number of

plant hosts species. In ambrosial *Geosmithia*, our result follows this pattern. They have preserved their enzymatic diversity allowing them to utilize nitrogen and phosphorus sources available in the xylem network and in beetle galleries in the form of excrements, exuviae and dead beetles (Meerts, 2002). Likewise in other ambrosia fungi (Kim et al., 2011; De Fine Licht and Biedermann, 2012), lignocellulose degradation is not linked with the ambrosial strategy in the genus *Geosmithia*. Hypothetically, lignocellulose degradation can cause the loss of protection provided by beetle galleries which possibly precluded the evolution of these enzymatic abilities in ambrosia fungi. Their specialization to vectors seems to reside in their production of large conidia with increased genome size and nutritive value (Veselská and Kolařík, 2015), and probably in the accumulation of oleic fatty acid in the mycelium. Other potentially important variables, such as protein content and essential amino-acids content, need to be tested. Ambrosial status was not a significant predictor in our lipid analysis due to the strong similarity of generalist sister species of ambrosial *G. microcorthyli*. Nevertheless, the high production of oleic acid (up to 50%) in these sister species was unique among generalists and may be considered a pre-adaption to ambrosial ecology. There are no data about the spectrum of essential fatty acids in bark beetles. However, animals, including weevils (Earle et al., 1967), cannot synthesize linoleic (18:2) and linolenic fatty acids (18:3) which are therefore considered essential. Kok (1979) detected both of these essential fatty acids in three ambrosial fungi associated with *Xyleborus* beetles, and they constituted up to 30% of all fatty acids. Davis et al. (2019) detected a similar profile of fatty acids in a beetle-associated *Leptographium*. In *Geosmithia*, we found only linoleic fatty acid (mean content of 25%) which was most abundant in pine specialists and least abundant in ambrosial species. Nevertheless, linoleic and linolenic fatty acids are biosynthetized via desaturation of oleic acid, a process also known in fungi (Buček et al., 2014). Production of fatty acids is strongly affected by fungal growth conditions and culture age (Gottlieb et al., 1968; Shimp and Kinsella, 1977; Stahl and Klug, 1996). Our inability to detect linolenic fatty acid in ambrosial *Geosmithia* could indicate their inability to synthesize it or unsuitable conditions for its production during fungal growth. Perhaps, some interaction between fungi and ambrosial beetles is necessary for its synthesis. Moreover, oleic and linoleic fatty acids are known necromones signaling the presence of dead insects. Most arthropods avoid these places (Sun and Zhou, 2013) and thus defend itself against predation and illness (Yao et al., 2009). However, for the ambrosial beetles *Trypodendron lineatum* and *Gnathotrichus* spp., oleic acid does not act as a potent repellent (Nijholt, 1980). Considering the high content of oleic fatty acid in ambrosial *Geosmithia*, we could reason that their ambrosia beetles might repudiate oleic acid as a warning signal. This could give them a competitive advantage because other insects identify their galleries as places which should be avoided.

5. Conclusion

In this study we demonstrate that several adaptive changes have occurred during the evolution of *Geosmithia* fungi living in symbiosis with bark beetles. The first one is the growth restriction to a limited range of substrates, accompanied by enzymatic streamlining in pine specialists. They have lost a broad spectrum of metabolic pathways that are present in *Geosmithia* generalists over the course of their long co-evolution with beetles. Narrowing of the diversity of metabolic pathways was also observed in another *Geosmithia* specialist, the pathogen *G. morbida* which causes necrosis in *Juglans nigra* phloem tissue. The shift from a saprotrophic to a pathogenic life strategy in this species triggered a secondary adaptive change – the alternation of the enzymatic apparatus to modify lignocellulose, the main component of the cell wall of woody plants, which we propose is one of its virulence factors. As the third adaptive change in *Geosmithia* evolution, we suggest an increase in the nutritive value of ambrosia fungi by conidial and DNA content enlargement (Veselská and Kolařík, 2015) and proportional augmentation of oleic fatty acid in mycelial dry weight. However, the role of oleic acid is unclear. We suppose that apart of its nutritive value, oleic acid could hide ambrosia beetle galleries from other insects, including parasites or parasitoids, providing ambrosia beetles with a competitive advantage. Another adaptation or pre-adaption widespread across all ecological groups of *Geosmithia* is the ability to ecologically suppress a diversity of microbes (including entomopathogens) and recycle nitrogen via effective utilization of urea and chitin.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2019.06.005.

Data archival location

DNA sequence data are available as a part of the Supporting online material. Other primary data will be deposited at Dryad upon acceptance.

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