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CHAPTER 2

Arbuscular mycorrhizal interactions of mycoheterotrophic *Thismia* are more specialized than autotrophic plants

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ABSTRACT

The belowground interaction between plants and arbuscular mycorrhizal (AM) fungi is one of the most widespread mutualisms on Earth. In general, plants and AM fungi exchange photosynthetically fixed carbon for soil nutrients, but occasionally non-photosynthetic plants obtain carbon from AM fungi. These mycoheterotrophic plants are suggested to have more specialized interactions than green plants, although comprehensive comparisons between their AM communities are lacking.

We used next-generation sequencing to compare the AM communities from five closely related mycoheterotrophic species of *Thismia* (Thismiaceae), surrounding green plants, and soil, sampled over the entire temperate distribution of *Thismia* in Australia and New Zealand. We observed that fungal communities are phylogenetically more similar within each functional group, suggesting a specific association pattern according to the plant trophic mode. Similarly, both types of plants presented more clustered fungal communities when compared to the fungal pool in the soil. Moreover, the fungal communities of mycoheterotrophic plants are phylogenetically more restricted than in green plants, independent of geographic origin.

Our findings demonstrate that these mycoheterotrophic plants target more narrow lineages of fungi, despite the larger fungal pool available in the soil, and thus they are more specialized towards mycorrhizal fungi than autotrophic plants.

Introduction

The interaction between arbuscular mycorrhizal (AM) fungi and over 80% of the land plants is one of the most widespread mutualism on Earth (Smith & Read, 2008). The AM fungi, abundant in most terrestrial ecosystems, are obligatorily associated with the roots of plants and act like extensions of plant root systems for increasing nutrient uptake, especially phosphorus. However, despite the ubiquity of the interaction, the mechanisms that control its above- and belowground diversity are not well understood (van der Heijden *et al.*, 1998).

Plant diversity and productivity are significantly influenced by the AM fungal diversity in the soil (van der Heijden *et al.*, 1998; Vogelsang *et al.*, 2006). A key component in plant productivity is photosynthetic fixation of inorganic carbon. It is this carbon that plants transfer to their mycorrhizal partners in exchange for soil nutrients (Smith & Read, 2008). Occasionally, plants lineages lose the ability to perform photosynthesis but maintain belowground links with mycorrhizal fungi. This phenomenon has long fascinated researchers, because in such systems, the expected outcome is that the fungi would also withdraw their participation in the interaction (Sachs & Simms, 2006). Instead, these non-photosynthetic plants, known as mycoheterotrophs, still harbour AM fungi growing in their roots (e.g. Leake 1994; Bidartondo *et al.* 2002; Merckx *et al.* 2012).

Mycoheterotrophy is an evolutionarily stable mode of life present in more than 20,000 plant species (Merckx, 2013). It is characterized by the absence of photosynthesis, in which plants obtain carbon via the mycorrhizal fungi associated with their roots. The only known way AM fungi obtain their carbon is through symbiosis with a photosynthetic plant. Thus, mycoheterotrophic plants must rely on established mutualisms between photosynthetic plants and AM fungi, becoming cheaters within three-partite interactions (Bidartondo, 2005a; Sachs & Simms, 2006). Mycoheterotrophy can occur (i) during a short period of the life cycle of a plant, subsequently replaced by an autotrophic mode of life such as in most orchids, many ferns and lycopods, (ii) during the entire life cycle of a plant such as in some orchids and monotropes, or (iii) simultaneously with autotrophy – partial mycoheterotrophy as in some orchids (Merckx, 2013). Thus, mycoheterotrophy can be seen as a dynamic interaction along a continuum of possible outcomes. Because mycorrhizal associations are generally mutualistic (Smith & Read, 2008), it is intriguing why, and which, fungi are part of a mycoheterotrophic interaction. In particular, the differences between mycorrhizal

associations of mycoheterotrophic and green plants, and potential preference towards particular fungal lineages, remains poorly known. Many mycoheterotrophic plants are known to have more specialized interactions towards basidiomycete fungi (i.e. they interact with fewer fungal lineages) than ectomycorrhizal green plants, presumably to increase their fitness by optimizing host adaptation (Cullings *et al.*, 1996; Bidartondo, 2005a). However, this pattern of increased specificity remains speculative for arbuscular mycoheterotrophic interactions, since comprehensive direct comparisons between AM interactions of mycoheterotrophic and green plants have not been reported. To investigate this, data about the mycorrhizal partners of mycoheterotrophic plants needs to be generated and compared to the fungal communities associated with green plants.

In the past years, the study of fungal diversity patterns has become more important in understanding the mechanisms driving plant biodiversity (Öpik *et al.*, 2009; Davison *et al.*, 2011; Peay *et al.*, 2013). Next-generation sequencing techniques to identify AM fungi allow assessments of the complex fungal communities in soil and plant roots (Toju *et al.*, 2014). However, species delimitation of the ancient and apparently strictly assexual AM fungi has long been debated and no consensus has been achieved for suitable molecular markers with sufficient resolution for species-level identification, nor for the cut-off values to be used in clustering operational taxonomic units for species prediction (Bruns & Taylor, 2016). Thus, measuring species richness with standard methods may introduce a bias in the assessment of fungal communities' composition. To better understand how communities are structured, an integration of phylogenetic structure, trait information and community composition can offer relevant insights on the evolutionary and ecological processes shaping communities (Webb *et al.*, 2002). At the community scale, species should be segregated based on relative strengths of habitat filtering and competition among similar species. Community structure can be phylogenetically clustered, random, or over-dispersed on the phylogeny of the entire available pool of species. For example, Kembel & Hubbell (2006) have found that phylogenetic structure of rainforest tree communities varied among habitats in Panama. They found communities with more closely related taxa than expected by chance (phylogenetically clustered), suggesting a stronger habitat filtering as the driving force of community assemblages, while other communities were composed by more distant taxa (overdispersion), suggesting current or past competitive exclusion between closely related taxa, or convergent evolution of important traits for persistence in such habitats.

In this study, we consider a community to be composed by fungal OTUs belonging to the same trophic level and the same guild (AM fungi: mycorrhizal fungi from the Glomeromycota phylum) co-occurring spatially in the roots of a plant. We compare the phylogenetic structure of the fungal communities associated with *Thismia* plants and co-occurring green plants (comparing plant nutrition types: mycoheterotrophic and autotrophic) confined to the distribution area of the selected mycoheterotrophic lineage, by studying the fungal community composition in their roots using high-throughput DNA sequencing methods. We focus on the temperate mycoheterotrophic *Thismia* species to evaluate the mycorrhizal associations patterns within an entire mycoheterotrophic plant clade. Because specificity in biotic interactions may differ considerably over a species' distribution range (Thompson, 2005), we study the interactions over the geographic range of this *Thismia* clade. Soil samples were included to estimate the fungal pool available for these species. To evaluate general differences in fungal community structure between mycoheterotrophic and autotrophic plants, we use phylogenetic measures to infer community structure.

Materials and Methods

Sampling

We sampled temperate forest sites in Australia and New Zealand over the known distribution of the *Thismia* clade in the region. At each site, one to five *Thismia* specimens where sampled, at least 1m from each other. This resulted in sampling 18 sites within three broad areas: 4 in New South Wales (NSW), 10 in Tasmania (TAS) and 4 in New Zealand (NZ).

At each site, the entire root system of *Thismia* and root tips (c. 1 cm) of surrounding plants where taken and preserved on CTAB buffer. The sampling of the surrounding green plants was done by selecting up to eight root tips of green plants found in the same soil clump as *Thismia*. To estimate the fungal pool available for all plant species, soil was sampled from the soil clump as well. Soil was dried on silica gel before DNA extraction. The sampling effort resulted in 99 samples, including MH, green plants and soil (Supporting Information Table S1). All plant roots were identified using molecular methods (Supporting Information Methods S1).

Assessment of fungal communities using Ion Torrent

Fungal DNA was extracted from the CTAB preserved roots with the KingFisher Flex Magnetic Particle Processor (Thermo Scientific, USA) using the NucleoMag 96 Plant Kit (Machery-Nagel, Germany). Subsequently, amplicon libraries were created to amplify the internal transcribed spacer (ITS2), using the fungal specific primer fITS7 (Ihrmark *et al.*, 2012) and ITS4 (White *et al.*, 1990) with a unique MID (Multiplex Identifier) label per sample, following the protocol described in Ihrmark *et al.* (2012). Sequencing was performed with a Personal Genome Machine (Ion Torrent; Life Technologies, Guilford, CT, USA) with 850 flows. Sequences obtained were processed using the UPARSE algorithm (Edgar 2013) incorporated in USearch v.7 (http://www.drive5.com/usearch/). Fastq files were screened for quality control and trimmed at the first base with Phred score of $Q < 20$. Followed by derreplication, singletons and sequences with less than 100 bp filtered out, the resulting sequences were clustered into OTUs at 97% similarity (Blaalid *et al.*, 2013). The taxonomy was assigned to the OTUs with Upparse, based on the UNITE + INSD database (10.09.2014) implemented with the current Index Fungorum identification. Only OTUs belonging to the Glomeromycota were kept for further analysis. The rawdata were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under the accession SRP083901. Because of the imbalanced number of specimensobtained for mycoheterotrophic and green plants, we calculated the species richness estimate Chao2 (Chiu *et al.*, 2014) for each plant group, using the function specpool in the vegan R package (Oksanen *et al.*, 2015).

Fungal community dissimilarities among samples

We calculated the phylogenetic relatedness between the OTUs to measure community differences between samples. An alignment of the OTU sequences and several reference Glomeromycota taxa from (Krüger *et al.*, 2012) was constructed with Mafft (Katoh & Standley, 2013). Phylogenetic inference on the OTU sequences was performed with RaxmlHPC-SSE3 (Stamatakis, 2014) using the GTR+G+I model of substitution as determined by jModeltest v2.1.5 using the Akaike information criterion (AIC; Darriba *et al.*, 2012). The phylogenetic distances among fungal OTUs given the highest-likelihood tree were used to obtain a fungal community dissimilarity matrix between all the pairs of samples, using the function Comdist in the picante R package (R Development Core Team, 2008; Kembel *et al.*, 2010). This algorithm finds for each fungal OTU in

one sample the average distance to all the OTUs in the other sample, and calculates the mean of these phylogenetic distances. The fungal community dissimilarities were visualized by performing a METAMDS in the VEGAN R package (Oksanen *et al.*, 2015). We investigated whether these fungal community dissimilarities differed between the 'type' of material (MH: mycoheterotrophic plants; green: green plants; and soil) and 'region' (New South Wales, Tasmania and New Zealand) with a permutational MANOVA using the function ADONIS in the VEGAN R-package (Oksanen *et al.*, 2015).

In addition, we explored whether the community dissimilarity patterns observed in the *Thismia* species were correlated with the plant evolutionary relationships. For that, we computed the Mantel test correlation between the fungal community dissimilarity matrix and the phylogenetic distance matrix among the *Thismia* species (see Supporting Information Methods S2 for detailed methods).

Fungal phylogenetic community structure

To investigate the fungal community structure, we calculated the phylogenetic community structure indices developed by Webb (2000) for community assessment of rainforest trees, which have also been successfully applied for fungal community studies (e.g. Peay *et al.* 2010; Maherali & Klironomos 2012). The net relatedness index (NRI) and the nearest taxa index (NTI) measure the degree of phylogenetic clustering of a group of taxa over the whole pool of taxa in a phylogenetic tree or within particular terminal clades, respectively. Positive values indicate that the fungal OTUs are more closely related to one another than expected by chance (phylogenetic clustering), and negative values indicate that the fungal OTUs are more distantly related (phylogenetic evenness). The NRI measures the overall clustering across the phylogeny using the average pairwise distance of all taxa from a community. NRI is then equal to $1 - (MPD_{observed} - MPD_{random})/SD(MPD_{random})$, where MPD stands for mean phylogenetic distance, which measures phylogenetic distance among taxa using the pairwise branch lengths distances. The pairwise phylogenetic distances among the fungal taxa were obtained from the fungal OTUs phylogeny. Numerically, NRI is the inverse of the standardized effect size of the MPD, which compares the average phylogenetic relatedness in the observed and null communities, under a null model of randomizations, standardized by the standard deviation (SD) of phylogenetic distances in the null community (Webb *et al.*, 2002). We obtained 999 randomizations shuffling the tips of the phylogeny from the total pool of fungal taxa. The NTI measures the terminal clustering among the taxa from the

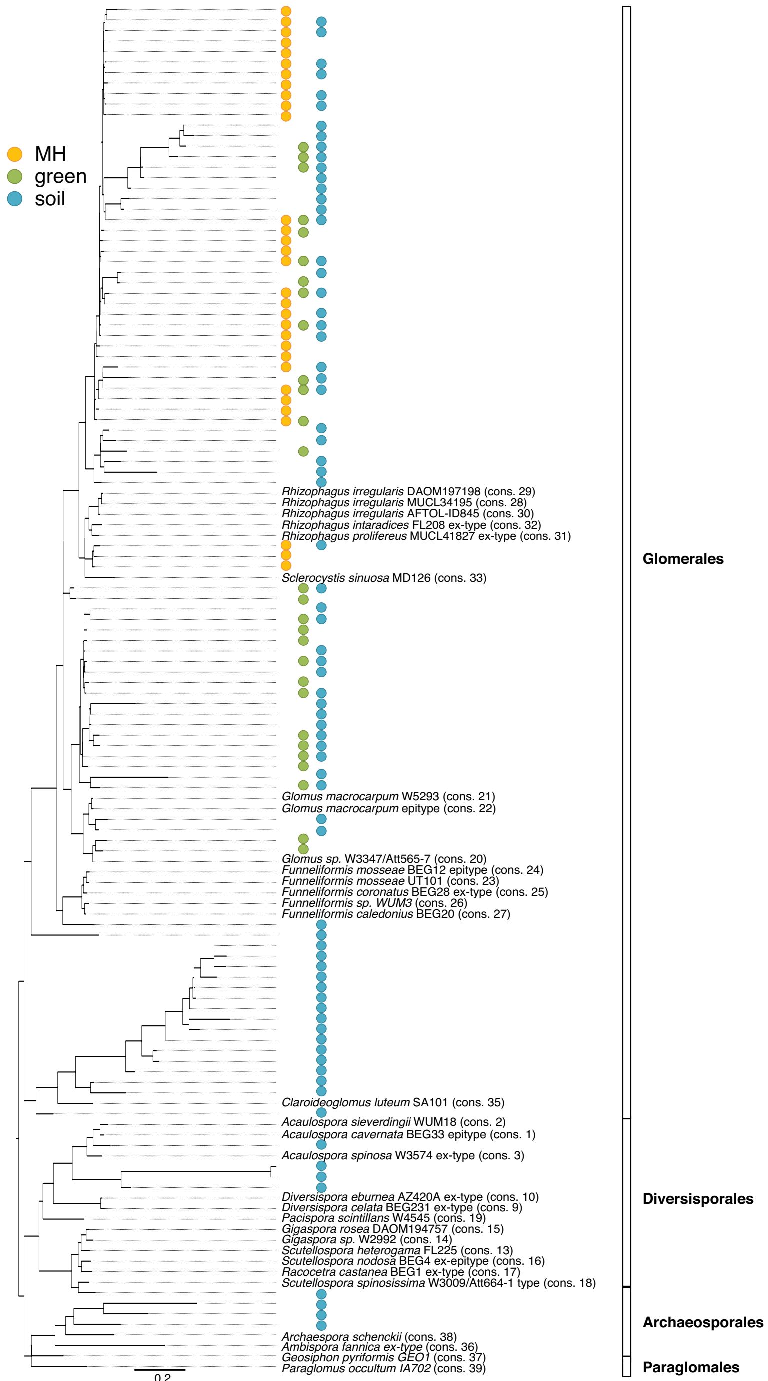
community. NTI is then equal to 1 – $(MNTD_{observed} - MNTD_{random})/SD(MNTD_{random})$, where MNTD stands for mean nearest phylogenetic taxon distance, which measures the minimal distance separating each species in the community. Numerically, NTI is the inverse of the standardized effect size of the MNTD, calculated similarly as MPD (Webb *et al.*, 2002). The standardized effects of the MPD and MNTD measures were calculated using PICANTE R-package (Kembel *et al.*, 2010).

In addition, for *Thismia* we reconstructed the NRI value of themost recent common ancestor of the clade based on the plant phylogenetic tree (pruned to contain only one taxon per species) and NRI values per species. Reconstruction was performed using phylogenetic independent constrasts (Felsenstein, 1985) as implemented in APE (Paradis *et al.*, 2004).

General patterns of fungal community structure

Because we were interested in general patterns of community structure, such as the specificity of interactions per trophic strategy, we focused on the NRI for an overall view of community clustering along the phylogeny. The observation of an overall phylogenetic clustered pattern indicates more specialized interactions, where the targeted fungal OTU taxa are more closely related than expected by chance. An overall phylogenetic overdispersion pattern suggests that the interactions are more generalist,where the targeted taxa are more spread out over the phylogenetic tree than expected by chance. In order to test the effects of the 'type' of material (mycoheterotrophic, green plants, and soil) on the NRI, we constructed a linear mixed-effects model with NRI as the response variable and 'type' of material as the predictor variable. We considered 'region' as a random factor to account for the nonindependence of the collections within and across regions. We then used a post hoc pairwise comparison test (Tukey's honest significant difference (HSD)) to assess whether the three types of material differed significantly from each other in their NRI.

Figure 1 (next page) | Highest likelihood tree (*LnL* = 10519.28) showing the phylogenetic relationships among the Glomeromycota operational taxonomic units (OTUs) found in all the samples, including several reference sequences. The colored circles indicate the presence of the fungal OTUs according to plant group (mycoheterotrophic, yellow; autotrophic, green) and the pool of fungal OTUs present in the soil (blue). Mycoheterotrophic plants of the genus *Thismia* are associated with fungi in the Glomerales family (one subclade: *Rhizophagus/Sclerocystis* sp.); and green plants are also associated with fungi in the Glomerales family (two subclades: *Rhizophagus / Sclerocystis* sp. and *Glomus* sp.). The soil also harbors fungi from the Glomerales family, and also from the Diversisporales and Archaeosporales families within the Glomeromycota phylum.


MH
green
soil
Rhizophagus irregularis DAOM197198 (cons. 29)
Rhizophagus irregularis MUCL34195 (cons. 28)
Rhizophagus irregularis AFTOL-ID845 (cons. 30)
Rhizophagus intaradices FL208 ex-type (cons. 32)
Rhizophagus prolifereus MUCL41827 ex-type (cons. 31)
Sclerocystis sinuosa MD126 (cons. 33)
Glomus macrocarpum W5293 (cons. 21)
Glomus macrocarpum epitype (cons. 22)
Glomus sp. W3347/Att565-7 (cons. 20)
Funneliformis mosseae BEG12 epitype (cons. 24)
Funneliformis mosseae UT101 (cons. 23)
Funneliformis coronatus BEG28 ex-type (cons. 25)
Funneliformis sp. WUM3 (cons. 26)
Funneliformis caledonius BEG20 (cons. 27)
Claroideoglomus luteum SA101 (cons. 35)
Acaulospora sieverdingii WUM18 (cons. 2)
Acaulospora cavernata BEG33 epitype (cons. 1)
Acaulospora spinosa W3574 ex-type (cons. 3)
Diversispora eburnea AZ420A ex-type (cons. 10)
Diversispora celata BEG231 ex-type (cons. 9)
Pacispora scintillans W4545 (cons. 19)
Gigaspora rosea DAOM194757 (cons. 15)
Gigaspora sp. W2992 (cons. 14)
Scutellospora heterogama FL225 (cons. 13)
Scutellospora nodosa BEG4 ex-epitype (cons. 16)
Racocetra castanea BEG1 ex-type (cons. 17)
Scutellospora spinosissima W3009/Att664-1 type (cons. 18)
Archaespora schenckii (cons. 38)
Ambispora fannica ex-type (cons. 36)
Geosiphon pyriformis GEO1 (cons. 37)
Paraglomus occultum IA702 (cons. 39)
0.2
Glomerales
Diversisporales
Archaeosporales
Paraglomales

Results

Plant identification

We successfully obtained sequences from the roots of 60 specimens of five *Thismia* species, 24 specimens of 11 green plant species and 25 soil samples (see Table S1 for details). The *Thismia* species were identified as *Thismia clavarioides* K. R.Thiele, *Thismia hillii* (Cheeseman) N. Pfeiff., *Thismia megalongensis* C. Hunt, G. Steenbeeke & V. Merckx, *Thismia rodwayi* F. Muell., and a fifth species that remains to be described, here termed *Thismia* sp. For the green plants, we identified the following species (Methods S1): Apocynaceae sp.; *Laurelia novae-zelandiae* A. Cunn., and *Doryphora sassafras* Endl. (Atherosper-mataceae); Bignoniaceae sp.; *Ceratopetalum apetalum* D. Don (Cunoniaceae); *Beyeria viscosa* Labill. (Euphorbiaceae); *Acacia* sp.(Fabaceae); *Beilschmiedia tawa* (A. Cunn.) Kirk (Lauraceae); *Pomaderris apetala* Labill. (Rhamnaceae); *Nematolepis* sp. Turcz. (Rutaceae); and Vitaceae sp. (Table S1). The success rate of sequencing Glomeromycota fungi from the autotrophic plants was considerably lower than for *Thismia*, and for several surrounding root samples we failed to obtain Glomeromycota OTUs. Some of the autotrophic plants are putatively ectomycorrhizal, which may explain the absence of Glomeromycota OTUs in surrounding roots. *Pomaderris apetala* and *Acacia* sp. can be both ectomycorrhizal and AM, and all other species are described as AM (Brundrett, 2008), except for *Beyeria viscosa* and *Nematolepis* sp. for which the mycorrhizal status is unknown, making them suitable for the comparisons in the downstream analysis.

Fungal sequences

Ion Torrent sequencing produced 4 038 169 raw sequences, of which 3 836 916 passed the quality filtering. After the quality control steps, the resulting sequences were clustered at 97% similarity, generating 466 OTUs, of which 99 OTUs were assigned to Glomeromycota and kept for subsequent analysis. Of these, 31 OTUs were found in the mycoheterotrophic plants, 28 OTUs were found in the green plants and 69 OTUs were found in the soil. The number of OTUs was not linearly correlated to the variable number of reads per sample, and thus neither is it linearly correlated to the number of OTUs per type of material (see Fig. S2). Using the Chao2 estimator, we obtained richness estimates of 32.26 ± SD 1.77 for mycoheterotrophic plants, 36.61 ± SD 6.30 for green plants, and 101.67 ± SD 15.12 for soil. Fig. 1 shows the highest likelihood phylogeny among the fungal OTUs and respective presence in mycoheterotrophic

plants, green plants andsoil. The fungal communities of the five *Thismia* species included Glomeromycota in the *Rhizophagus/Sclerocystis* sp. subclade; for the green plants, the same clades of fungi were present with the addition of the *Glomus* sp. subclade; in the soil, Glomerales, Diversisporales and Archaeosporales fungi were present.

Fungal community dissimilarities

Fungal community dissimilarities were calculated among all the samples, including mycoheterotrophic plants, green plants and soil. In Fig. 2, a nonmetric multidimensional scaling plot shows an ordination of the fungal community dissimilarities. Furthermore, we found no phylogenetic signal on the fungal community dissimilarities among the different *Thismia* species (Mantel test: $r = 0.092$; $P = 0.196$). Thus, we proceeded with the fungal community dissimilarity analysis including the green plants and looked for patterns within the 'type' of material (mycoheterotrophic plants, green plants and soil), and we also looked for geographic patterns ('region': Tasmania, New South Wales and New Zealand). Permutational MANOVA (Adonis) showed significant fungal

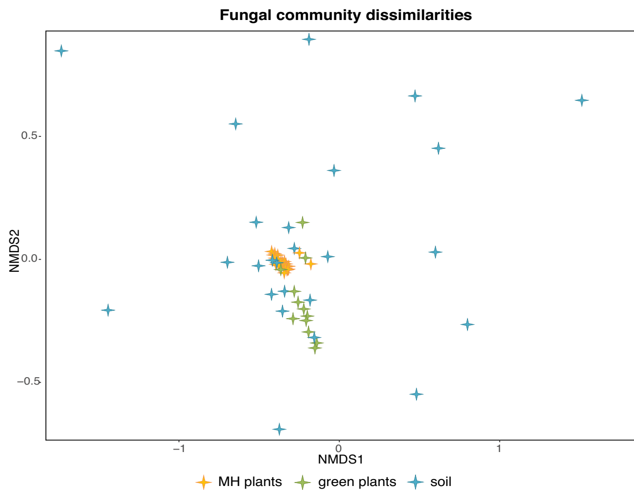


Figure 2 | Nonmetric multidimensional scaling plot (metaMDS) showing an ordination of the fungal community dissimilarities (Comdist) among all the samples. The fungal community dissimilarities are calculated based on the average phylogenetic distance between each fungal operational taxonomic unit (OTU) in one sample and the total OTUs in the other sample. Each symbol represents the Comdist of the fungal communities including all the OTUs found in each species per site. Permutational MANOVA (Adonis) showed significant fungal community dissimilarity between mycoheterotrophic (MH) *Thismia* plants, green plants, and soil ($F = 25.4$; $R^2 = 0.486$; $P = 0.001$).

Table 1 | Net relatedness index (NRI) and nearest taxa index (NTI) results for the fungal communities of mycoheterotrophic (MH) plants (*Thismia*), green plants and soil.

Type	Samples	N	NRI	RGR	NTI	RGR
MH plants	*T. rodwayi* 1 TAS	12	4.14**	999	2.38**	999
	T. rodwayi 2 TAS	4	2.16**	996	1.77**	996
	T. rodwayi 3 TAS	9	3.42**	999	2.13**	999
	T. rodwayi 4 TAS	14	4.49**	999	2.44**	999
	T. rodwayi 5 TAS	12	4.13**	999	2.34**	999
	T. rodwayi 6 TAS	3	1.79**	999	1.65**	998
	T. rodwayi 7 TAS	8	3.23**	999	2.12**	999
	T. rodwayi 8 TAS	9	3.41**	999	2.14**	999
	T. rodwayi 9 TAS	12	4.20**	999	2.41**	999
	T. rodwayi 10 TAS	8	3.24**	999	2.09**	999
	T. clavarioides NSW	5	2.53**	999	1.72**	995
	Thismia sp. NSW	3	1.80**	997	1.55**	998
	Thismia sp. NSW	4	1.74**	979	1.44**	957
	T. hillii NSW	7	3.00**	999	2.08**	999
	T. megalongensis NSW	6	2.67**	999	2.08**	999
	T. hillii 1 NZ	9	3.29**	999	2.17**	999
	T. hillii 2 NZ	4	1.95**	991	1.65**	994
	T. hillii 3 NZ	6	2.68**	998	2.04**	999
	T. hillii 4 NZ	6	2.61**	999	1.96**	999
green plants	*Acacia* sp. TAS	2	0.92	818	0.92	808
	Beyeria viscosa TAS	2	0.53	567	0.54	545
	Pomaderris apetala TAS	4	1.13	788	1.11	840
	Nematolepis sp. TAS	2	1.24*	919	1.21*	935
	Acacia sp. NSW	2	1.20	892	1.19	868
	Bignoniaceae sp. NSW	3	1.66**	986	1.52**	975
	Ceratopetalum apetallum NSW	3	1.02	774	0.97	788
	Doryphora sassafras NSW	10	2.97**	999	1.70**	975
	Vitaceae sp. NSW	7	2.15**	988	1.44*	921
	Apocynaceae sp. NSW	6	1.30	887	0.84	739
	Beilschmiedia tawa NZ	3	0.75	656	0.55	624
	Laurelia novae-zelandiae NZ	13	2.28**	983	1.93**	993
Soil	Soil 1 TAS	9	0.06	523	-0.91	181
	Soil 2 TAS	2	1.19	890	1.19	892
	Soil 3 TAS	2	-1.13	153	-1.14	157
	Soil 4 TAS	6	2.65**	999	2.07**	999
	Soil 5 TAS	11	-1.26	105	-1.85	38
	Soil 6 TAS	5	0.59	735	0.46	669
	Soil 7 TAS	2	-1.43	69	-1.46	67
	Soil 8 TAS	3	0.74	679	0.84	777
	Soil 9 TAS	6	1.16	829	0.67	722
	Soil 10 TAS	9	-1.86	30	2.02**	994
	Soil 11 TAS	2	1.32**	993	1.39**	995
	Soil 12 TAS	13	-0.26	385	1.86**	982
	Soil 13 TAS	14	-0.92	184	1.04	855
	Soil 14 TAS	2	-1.49	70	-1.52	71
	Soil 15 TAS	7	0.65	717	0.61	709
	Soil 16 TAS	4	2.20**	996	1.77**	997
	Soil 17 TAS	5	0.79	757	0.57	717
	Soil 18 TAS	3	0.04	561	0.46	648
	Soil 1 NSW	4	-0.67	273	-0.44	312
	Soil 2 NSW	16	-0.38	376	2.36**	998
	Soil 3 NSW	3	1.34*	933	1.36*	945
	Soil 4 NSW	3	1.65**	971	1.50**	993
	Soil 5 NSW	2	0.33	496	0.30	474
	Soil 6 NSW	3	0.80	667	0.95	812
	Soil NZ	6	1.30	864	1.29	893

Samples, species per site; *n*, number of OTUs in a community; *RGR*, number of times the observed NRI or NTI was greater than the value obtained for the random permuted communities.

*Communities significantly structured at the $P = 0.10$ level.
*Communities significantly structured at the $P = 0.10$ level.

community dissimilarity for 'type' of material (F = 25.4; R^2 = 0.486; P = 0.001), but not for 'region' (F = 0.925; R^2 = 0.018; P = 0.427). These results suggest a distinctive andspecific association pattern of the fungal communities for mycoheterotrophic plants, green plants and soil, regardless of the region in which they occur.

Fungal phylogenetic community structure

We observed that all the mycoheterotrophic plants exhibited positive and significant NRI and NTI values (Table 1), which indicates a significant phylogenetic structure of the fungal communities. The two indices were correlated (Fig. S3). By contrast, most of the green plants and soil communities were phylogenetically randomly structured for both indices (Table 1). The roots of mycoheterotrophic plants tended to be colonized by AM fungi that were more closely related than expected by chance. The green plants tended to show no clear pattern in general, except for five species that presented phylogenetic structure. The soil also seemed to be mostly randomly phylogenetically structured. Overall, the two indices were concordant. The NRI of the most recent common ancestor of the *Thismia* clade was reconstructed to be 4.00 (95% confidence interval (CI) 3.26–4.74; see Fig. S4).

General patterns of fungal community structure

The mixed-effects model results showed that the fungal community structure was significantly explained by the 'type' of material. The fungal communities associated with the mycoheterotrophicplants were significantly more closely related to each other than in the case of the green plants and the soil. Likewise, for the green plants, the fungal communities were also significantly more closely related to each other than in the soil (see Fig. 3; Table S2).

Discussion

The plant sampling was designed to investigate the fungal community structure of closely related mycoheterotrophic plant species over their entire geographic range and, at the same time, compare their fungal community structure with that of the surrounding autotrophic plants, as a proxy for mycoheterotrophic and autotrophic types of nutrition, respectively. The soil data were used as a proxy for the diversity of local AM

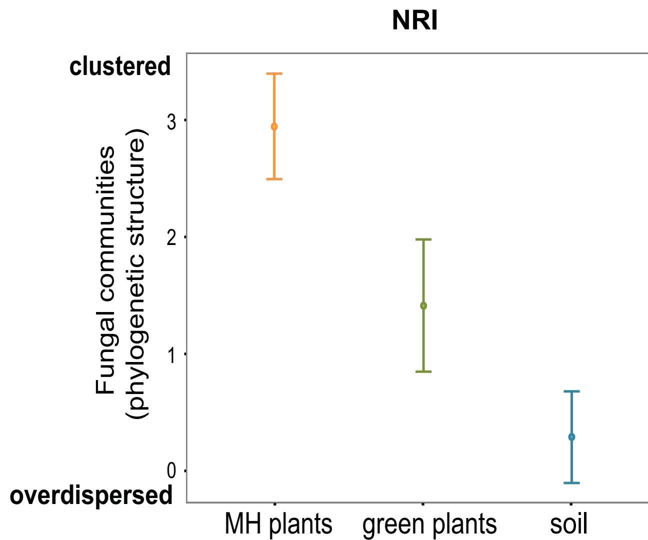


Figure 2 | Fungal community structure based on the net relatedness index (NRI) for each species per site. The graph represents the fungal communities' phylogenetic dispersion patterns as explained by the 'type'of material (mycoheterotrophic (MH) plants, green plants and soil). Negative NRI values indicate that the fungal communities are overdispersed in the phylogenetic tree, while positive NRI values indicate phylogenetic clustering. The NRI was significantly different in MH plants compared with green plants and soil. MH plants harbor more phylogenetically clustered AM fungal communities in their roots than green plants and the soil. Green plants also have significantly more clustered fungal communities than the soil. The mixed-effects model estimates with 95% confidence intervals are shown. See Supporting Information Table S2 for statistical details.

fungi. As expected, the soil presented a higher fungal diversity compared with individual plants, as it harbors the fungal reservoir from which the plant species obtain their fungal partners (Table S1). Our results indicate that, in general, mycoheterotrophic and green plants have distinct fungal community compositions with no geographic pattern (Fig. 2; Adonis test). In addition, the five closely related *Thismia* species tended to associate with more closely related AM fungi more often than expected by chance. Observations of other cases of mycoheterotrophic species growing on narrow phylogenetic lineages of AM fungi have been reported previously, for example *Arachnitis* (Bidartondo *et al.*, 2002), *Afrothismia* (Merckx & Bidartondo, 2008), *Burmannia* (Ogura-Tsujita*et al.*, 2013) and *Petrosavia* (Yamato *et al.*, 2011). Moreover, we observed that the phylogenetic structure of the fungal communities can vary according to the type of nutrition of aplant (i.e. mycoheterotrophic vs autotrophic; see Fig. 3).

For the mycoheterotrophic plants, we detected significant NRI and NTI values (Table 1). These two indices provide information about community structure that is

different from that provided by richness or taxonomic composition. In view of the unequal number of specimens of mycoheterotrophic and green plants and differences in sequencing success, we calculated the improved richness estimator CHAO2 of Chiu *et al.* (2014), incorporating a small sample correction. This estimator reduces the bias when the heterogeneity of species detection probabilities is relatively high (Chiu *et al.*, 2014). While the estimated richness was higher for the green plants than for the mycoheterotrophic plants, the observed richness was higher for the mycoheterotrophic plants. Considering phylogenetic relatedness among the taxa, we found that, within the Glomeraceae family, the fungi associated with mycoheterotrophic plants belonged to one subclade, while green plants had fungal partners in two subclades (Fig. 1). Thus, the higher estimated richness for the green plants corresponded to a higher phylogenetic diversity compared with the mycoheterotrophic plants.

The phylogenetic clustering pattern observed in the mycoheterotrophic plants' fungal communities reflected ecological rather than biogeographic patterns, as there was no geographical structure of the fungal communities. Moreover, the tendency of *Thismia* species to target the same narrow clades of AM fungi (Fig. S5), and their similar levels of mycorrhizal specificity (Table 1), also reconstructed to have been present in the most recent common ancestor of the clade (Fig. S4), strongly suggest that the high level of mycorrhizal specificity is prone to phylogenetic niche conservatism (Harvey & Pagel, 1991; Lord *et al.*,1995), that is, the tendency of these *Thismia* species to retain similar ecological traits (i.e. similar fungal communities) overtime (Wiens & Graham, 2005; Wiens *et al.*, 2010). The phylogenetic niche conservatism observed in *Thismia* may be attributable to a reduction in the potential range of ecological character evolution caused by fixation of ancestral traits, enabling the descendants within this plant lineage to be more successfully adapted inparticular and similar habitat types (Lord *et al.*, 1995). The reason for the preference for targeting certain lineages of AM fungi in this mycoheterotrophic interaction is still not well understood. It is certainly not caused by a limited local availability of AM fungi, because we detected a much larger and phylogenetically broader pool of available fungi in the soil. Similar to the explanation for the high host specificity of many parasites, the mycoheterotrophs may fine-tune their physiology on particular lineages of fungi to maximize their carbon uptake (Leake & Cameron, 2010). Alternatively, the mycoheterotrophic plants may be rejected by most fungal lineages in the pool of available fungi, and therefore the pattern would result from an evolutionary arms race (Bidartondo, 2005). Therefore, it is our interpretation that the fungal communities associated with these mycoheterotrophic plants might

have been shaped not only by habitat filtering (occurrence of the fungal partners in space), but also by an effect of the ancestry of the plant species, which allow this local third-party cheater (*Thismia*) to participate in the globally mutualistic AM interaction with autotrophic plants.

For the green plants, some species showed significantly phylogenetically clustered AM fungal communities (Table 1). Specific patterns in the fungal associations of green plants have been previously reported in other studies (e.g. Öpik *et al.*, 2009; Davison *et al.*, 2011; Peay *et al.*, 2013). Nonetheless, other green plants in our study presented a randomly assembled fungal community. This may reflect a different community structure according to plant species, but it may also be an effect caused by an underrepresentation of the fungal communities, which was more likely to occur in the green plants than in the mycoheterotrophic plants because of sampling method limitations. For the green plants we could only collect a few centimeters of the extensive root system, so, because of the scattered pattern of AM fungal colonization along the roots, we may have assessed a limited fraction of the whole diversity, while for the mycoheterotrophic plants, we collected the entire small root system. Nevertheless, we do not think that this underrepresentation of green plants' fungal communities introduced bias to our results, because although it could be assumed that we were observing partial diversity, we obtained less phylogenetic clustering in green plants than in mycoheterotrophic plants. The phylogenetic clustering of these communities would become even more diluted with the introduction of more phylogenetically different taxa in the analysis, and therefore the specificity would decrease (Webb, 2000). Generally, the comparison of fungal communities associated with mycoheterotrophic and autotrophic plants showed that this particular lineage of mycoheterotrophic *Thismia* species have significantly more specialized interactions than the green plants living in the same regions (Fig. 3). Mycoheterotrophic plants had significantly more specialized fungal interactions than green plants, because the mycoheterotrophs showed higher NRI values almost exclusively. Similarly, mycoheterotrophic plants also had generally higher ranks of NTI values (Table 1). This suggests that, within the Glomerales subclade targeted by mycoheterotrophic plants, these plants also tend to target specific lineages at a lower taxonomic level. These results support the view that mycoheterotrophic mycorrhizal interactions are highly specialized. By contrast, green plants did not always show significantly clustered patterns. If we excluded the green plants for which we detected fewer than three OTUs (minimum number of OTUs found in the *Thismia* species), we found that half of the autotrophic plants (*Doryphora sassafras*, Bignoniaceae sp., *Laurelia novae-*

zelandiae and Vitaceae sp.) tended to associate with more closely related main lineages of AM fungi than expected by chance, but generally with lower ranks of positive NRI and NTI values compared with *Thismia*. We also found that the other half (Apocynaceae sp., *Ceratopetalum apetalum*, *Beilschmiedia tawa* and *Pomaderris apetala*) did not present a significantly clustered pattern. In conclusion, even though some green plants may also tend to target more closely related AM fungal taxa than expected by chance, in general these green plants have less specialized interactions compared with *Thismia*. In this study, we tested the association between these two ecological traits (type of plant nutrition (mycoheterotrophic vs autotrophic) and phylogenetic fungal community structure) for these *Thismia* species and surrounding green plants. The study of fungal community structure needs to be extended to other distantly related lineages of mycoheterotrophic plants before we make generalizations about the processes shaping the fungal interactions involved in mycoheterotrophy. Moreover, understanding how the fungal communities associated with plants in general are assembled can provide us with knowledge of how belowground ecological processes influence the global distribution of plants in ecosystems.

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Author Contribution

S.I.F.G. and V.S.F.T.M planned and designed the research, V.S.F.T.M. collected the samples, S.I.F.G. generated the data and performed the analysis, J.A.-.G. participated in the data analysis, M.I.B. contributed to the interpretation of the data, S.I.F.G. wrote the manuscript. All the authors commented on the final version of the paper.

Supporting Information

Methods S1

2

The plants collected in this study consisted of mycoheterotrophic and green species. The mycoheterotrophic species were identified by the genetic markers *ITS*, using the primers ITS1 and ITS4 (White *et al.*, 1990) and *cob*, using the primers COB1F and COB1R (Petersen *et al.*, 2006; GenBank accessions KX790794–KX790923). Partial *matK* sequences were obtained from the root tips DNA extractions of the surrounding plants and leaf samples of identified species collected at the sites using primers trnK685F and matK1777R (Hu *et al.*, 2000). For several plant samples from sites in New South Wales this did not result in amplification products. For these plants partial *trnL* sequences were obtained with primers trnL-f and trnL-c (Taberlet *et al.*, 1991). Root tips were identified based on their sequence similarity with the leaf samples and / or BLAST searches on GenBank.

Figures

Figure S1 | Map of sampling localities. A total of 18 sites were sampled within three broad areas: 4 in New South Wales, 10 in Tasmania and 4 in New Zealand. Inset shows a flower of *Thismia rodwayi* as illustration of one of the species (bar, 1 cm).

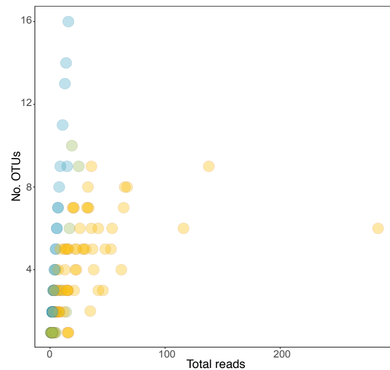

Figure S2 | Plot of the total number of OTUs against the total number of reads. The total number of reads originated by the Ion Torrent run after the quality control steps (excluding sequences with $Q < 20$) was plotted against the number of OTUs after clustering at 97% similarity, across all samples. Pearson correlation test (r = 0.31, $P < 0.05$) shows a weak correlation between the number of reads and the number of OTUs generated, but there is not a linear relationship ($r^2 = 0.096$, $P < 0.05$).

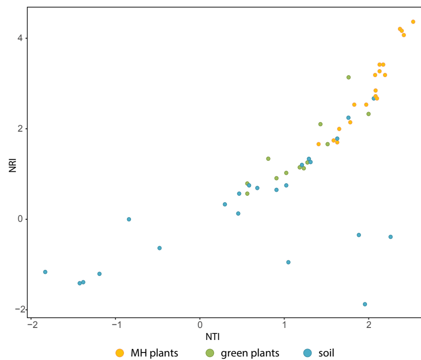

Figure S3 | Relationship between the net relatedness index and nearest taxa index. Pearson correlation test (r = 0.77, $P < 0.001$) shows that both indices (see Table 1) are correlated, indicating that an overall fungal communities' clustering or dispersal on the deeper nodes of the tree (NRI) corresponds to a similar extent of terminal clustering or dispersal, i.e., near the tips of the tree (NTI).

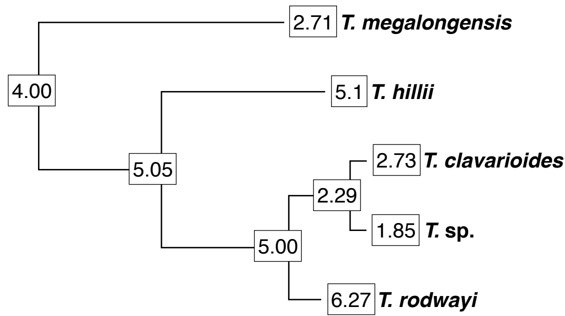

Figure S4 | Ancestral state reconstruction of the NRI on the species level *Thismia* phylogeny. For each species, the observed NRI is shown at the tips and the reconstructed values are shown on the nodes. The reconstructed NRI of the most common recent ancestor of this lineage (4.00; 95% CI: 3.26–4.74) is within the range of the extant species, which means that the ancestor had similar mycorrhizal specificity, and thus already showed specialized interactions.

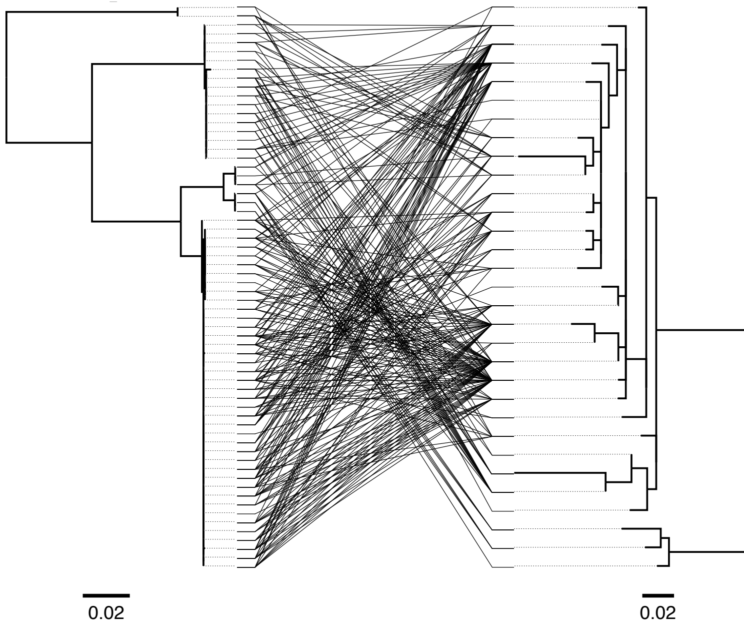

Figure S5 | Tanglegram of the interactions between mycoheterotrophic species of *Thismia* and AM fungal OTUs. The phylogenetic tree of *Thismia* is represented on the left side (see Supporting Information, Methods S2 for details on the phylogenetic relationships of the five species of *Thismia*), and the phylogenetic tree of the AM fungal OTUs on the right side (same as Fig. 1). The tanglegram was built using the APE R package. The figure shows extensive overlap in the fungal interactions within the five *Thimia* species.

Tables

Table S1 | Summary of the samples used in the analysis. In total, we found 99 Glomeromycota OTUs in 5 MH *Thismia* species and 11 green plant species. The table shows the number of samples (total 109), which were pooled in 61 samples for Ion Torrent sequencing. 'No. OTUs' corresponds to the number of unique OTUs found per plant species per site, or per locality in the case of soil samples. Species identification of the green plants is showed to the lowest taxonomical level possible to identify based on *matK* or *trnL* genetic markers.

Species	No samples	Pooled samples	Type	Location	No. OTUs
Thismia rodwayi	37	10	MH	Tasmania	23
Thismia megalongensis	2	1	MH	NSW	6
Thismia hillii	2	1	MH	NSW	7
Thismia clavarioides	2	1	MH	NSW	5
Thismia sp	3	3	MH	NSW	5
Thismia hillii	14	5	MH	NZ	16
Beyeria viscosa	1	1	green	Tasmania	2
Pomaderris apetala	4	1	green	Tasmania	4
Nematolepsis	1	1	green	Tasmania	2
Acacia sp	2	1	green	Tasmania	2
Ceratopetalum apetalum	2	1	green	NSW	3
Acacia sp	1	1	green	NSW	2
Doryphora sassafras	2	2	green	NSW	10
Bigoniaceae	1	1	green	NSW	3
Vitaceae	3	2	green	NSW	7
Apocynaceae	3	1	green	NSW	6
Beilschmiedia tawa	2	1	green	NZ	4
Laurelia novae-zelandiae	2	2	green	NZ	14
Soil	18	18	soil	Tasmania	56
Soil	6	6	soil	NSW	29
Soil	1	1	soil	NZ	7

Table S2 | Statistical results of the mixed-effects model and multiple comparison analysis explaining the fungal communities' phylogenetic dispersion patterns by the 'type' of material (MH plants, green plants, soil), using 'region' as a random factor. The multiple linear comparisons test whether the degree of phylogenetic dispersion of the fungal communities is significantly different among mycoheterotrophic plants, green plants and soil.

Comparisons	Coefficient	SE	P-value
MH plants	2.97	0.23	< 0.001
green plants	1.43	0.29	< 0.001
soil	1.30	0.20	0.433
MH plants - green plants	1.54	0.37	<0.001
MH plants - soil	2.68	0.31	<0.001
green plants - soil	1.13	0.36	0.007