

# The network structure of plant–arbuscular mycorrhizal fungi

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## Summary

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**Key words:** community assemblages, modularity, mutualism, nestedness, network, plant–arbuscular mycorrhizal fungi (AMF) association.

- Ecological network theory predicts that in mutualistic systems specialists tend to interact with a subset of species with which generalists interact (i.e. nestedness). Approaching plant–arbuscular mycorrhizal fungi (AMF) association using network analyses will allow the generality of this pattern to be expanded to the ubiquitous plant–AMF mutualism.
- Based on certain plant–AMF specificity recently suggested, networks are expected to be nested as a result of their mutualistic nature, and modular, with certain species interacting more tightly than others. Network analyses were used to test for nestedness and modularity and to compare the different contribution of plant and AMF to the overall nestedness.
- Plant–AMF networks share general network properties with other mutualisms. Plant species with few AMFs in their roots tend to associate with those AMFs recorded in most plant species. AMFs present in a few plant species occur in plant species sheltering most AMF (i.e. nestedness). This plant–AMF network presents weakly interlinked subsets of species, strongly connected internally (i.e. modularity). Both plants and AMF show a nested structure, although AMFs have lower nestedness than plants.
- The plant–AMF interaction pattern is interpreted in the context of how plant–AMF associations can be underlying mechanisms shaping plant community assemblages.

## Introduction

The association between arbuscular mycorrhizal fungi (AMFs) and plants improves the fitness of both plant and AMF symbionts, constituting a traditionally considered mutualism (Blackwell, 2000). AMF increase plant uptake of soil nutrients, especially phosphorus (P) (Smith & Read, 1997), while the plants provide carbon compounds to the AMF, although, in some cases, the equitability of resource exchange between plants and AMF might not be mutually beneficial (Johnson *et al.*, 1997). A more efficient nutrient uptake as a result of AMF associations can alleviate plant competition for mineral resources (Fitter, 1977; Allen & Allen, 1984; Hetrick *et al.*, 1989; Moora & Zobel, 1996; Bever *et al.*, 1997). An equitable distribution of soil resources among competitively dominant and subdominant host species might promote plant species coexistence (Walter *et al.*, 1996; Malcová *et al.*, 1999). Plant species associations with specific AMF taxa can ultimately influence AMF community composition (Grime *et al.*, 1987; Van der Heijden *et al.*, 1998a,b; Hartnett & Wilson, 1999) and bottom-up influence of AMFs on plant community diversity has also been reported (Grime *et al.*, 1987; Van der Heijden *et al.*, 1998a,b; Hartnett & Wilson, 1999), potentially mediated by

plant-to-plant facilitation (Van der Heijden & Horton, 2009). Facilitation is a key process structuring plant communities in semiarid regions, where P soil availability can be limiting (Cross & Schlesinger, 2001; Li *et al.*, 2004). In this P-limiting environment, 97% of the plant species require other plant species to recruit successfully, and 57% of these positive interactions are maintained when the plants reach the adult stage (Verdú & Valiente-Banuet, 2008). Elucidating the plant–AMF interaction pattern is a first step to exploring the potential mechanism underlying plant-to-plant facilitation and its implications in structuring plant community assemblages.

Regardless of the importance of mycorrhizal associations, which form associations with most of land plants (Wang & Qiu, 2006; Smith & Read, 2008; Brundrett, 2009), the pattern of plant–AMF interactions still remains largely unknown in natural communities (Bever, 2003; Van der Heijden & Horton, 2009). In order to evaluate the plant–AMF interaction pattern in a community, it is necessary to sample a representative number of plant species growing in the same area and exposed to the same AMF taxon pool (Davison *et al.*, 2011). The availability of studies presenting representative sampling of plants and AMF communities in a given natural site is scarce. Several studies have characterized the diversity of fungal communities in natural environments by focusing on a few – usually the most common – plant species

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rhizospheres in the community (Daniell *et al.*, 2001; Zhaoyong *et al.*, 2006; Kottke *et al.*, 2008; Alguacil *et al.*, 2009; Sonjak *et al.*, 2009; Wilde *et al.*, 2009; Öpik *et al.*, 2010 for further references) or by exploring the influence of the plant community on final AMF composition (Mummey *et al.*, 2005; Hausmann & Hawkes, 2009) using artificial (i.e. experimental) communities (Van der Heijden *et al.*, 1998b; Maherali & Klironomos, 2007). However, very few studies aim at sampling most of the plant and AMF communities in natural environments in order to elucidate the pattern of plant–AMF interactions (but see Öpik *et al.*, 2009; Davison *et al.*, 2011). Thus, it is still largely unclear to what extent plant and AMF communities interact in a random way or, alternatively, if biological processes can lead to emerging nonrandom plant–AMF interaction patterns.

Arbuscular mycorrhizal fungi belong to the phylum Glomeromycota, one of the key taxa interconnecting plants into a functional web (Helgason *et al.*, 1998). Despite the fact that Glomales form symbiotic associations with the majority of land plants (65–85%; Wang & Qiu, 2006; Smith & Read, 2008; Brundrett, 2009), < 200 species of these globally important fungi have been described (Morton & Benny, 1990). The apparent low diversity of AMFs compared with their associated plant hosts has led to the historical presumption that plant–AMF associations must have a low specificity (Smith & Read, 1997). Nonetheless, it is becoming increasingly clear that distinct AMF communities are present in the rhizosphere (Bever *et al.*, 1996; Eom *et al.*, 2000) and that there is a certain specificity in the interaction with plant species (Helgason *et al.*, 2002; Vandenkoornhuysen *et al.*, 2002, 2003; Scheublin *et al.*, 2004; Pivato *et al.*, 2007; Santos-González *et al.*, 2007; Mummey & Rillig, 2008; Öpik *et al.*, 2008; Smith & Read, 2008; Li *et al.*, 2010; Davison *et al.*, 2011). This shift has been influenced by the greater AMF diversity revealed by the use of molecular techniques, with higher resolution for distinguishing closely related species. However, the different amount of intraspecific genetic variation depending on the family, genus and species prevents the determination of a generalized genetic threshold to delimitate AMF species (Redecker *et al.*, 2003; Rosendahl, 2007; Nilsson *et al.*, 2008). The increasing knowledge about AMF diversity and availability of molecular tools to measure it, offers a unique opportunity to explore plant–AMF interaction patterns in natural communities.

Network analysis is a convenient technique to detect non-random species interaction patterns. This analysis has been used to study different types of mutualisms: plant–pollinators and seed dispersers (Bascompte *et al.*, 2003), marine cleaning mutualisms (Guimarães *et al.*, 2007) or plant-to-plant facilitation (Verdú & Valiente-Banuet, 2008). However, network analyses have been rarely applied to fungal communities (but see Peay *et al.*, 2007 and Vacher *et al.*, 2008) and, as far as we know, have not been previously applied to study plant–AMF interactions at the community level. The wide application of network analyses has led to the development of an ecological network theory based on emerging patterns shared by multiple mutualistic systems. Interestingly, networks representing mutualistic processes have been shown to share a well-defined network structure regardless of the nature of the species involved (Bascompte & Jordano, 2007 but

see Joppa *et al.*, 2010 for a methodological critique). Ecological network theory predicts that mutualistic networks are characterized by having a few species much more connected than is expected by chance, in which specialists tend to interact with a subset of the species with which generalists interact (i.e. nestedness) (Bascompte & Jordano, 2007). This particular structure has implications for the robustness of the network and coexistence and stability of species (Bascompte & Jordano, 2007). In addition, if plant–AMF interactions are not as generalist as traditionally thought, it can be expected that any pair of species do not necessarily have the same probability of interacting. Accordingly, a group of plant species will tend to interact predominantly with a given group of AMFs, and vice versa. Network modularity reflects the tendency of a set of species to interact predominantly with species within the set and less frequently with species in other sets. Modularity implies that species can be grouped (i.e. modules) in such a way that weakly interlinked subsets of species are strongly connected internally (Olesen *et al.*, 2007). Approaching the study of plant–AMF interaction from a network perspective will provide the opportunity to test two hypotheses: first, that the plant–AMF interaction pattern matches the predictions developed by network theory based on other mutualistic systems; and second that the nonrandom plant–AMF interactions occur at the community level when most of the plant community is considered.

In this study we characterize the interaction patterns in a plant–AMF mutualistic system. For the sake of generality, we reanalyse, using network analyses, the data from the two available studies which sampled most of the plant community and recorded higher AMF phylogenetic diversity than our study. We define a gradient of AMF genetic differentiation threshold values (hereafter cutoff) and, for each one, first describe the network testing for nestedness and modularity, and then compare the different contributions of plants and AMFs to the overall nestedness. Finally we estimate the relative contribution of plant species abundance to the observed interaction pattern. We show how this mutualism fits into mutualistic network theory and previous knowledge about plant–AMF interactions, discussing the potential implications for plant community structure mediated by plant-to-plant facilitation.

## Materials and Methods

### Study area and plant sampling

This study was conducted in the semiarid Valley of Zapotitlán (18°20'N, 97°28'W), a local basin of the biosphere reserve of Tehuacán–Cuicatlán Valley in the state of Puebla, Mexico. This region owes its aridity to the rain shadow produced by the Eastern Sierra Madre (Valiente-Banuet *et al.*, 2000). It has an annual average rainfall of 380 mm, most of which falls during the summer months, and an annual mean temperature of 21°C with rare frosts (García, 1973). Specifically, the study site is located c. 30 km south of Tehuacán city in a natural area in which vegetation is a xeric shrubland (woody perennial species) dominated by the columnar cactus *Neobuxbaumia tetetzo*, *Agave* spp., different Fabaceae and Asteraceae species, among other taxa. The

vegetation is characterized by individuals of multiple species spatially associated forming discrete vegetation clumps, although some isolated individuals can also be found. Vegetation clump areas range from 1 to 5 m<sup>2</sup>.

Phosphorus concentration in soils at each vegetation clump is very low, ranging from 2 to 19 mg kg<sup>-1</sup>, mean  $\pm$  SE = 5.37  $\pm$  0.44 (L. Sortibrán, unpublished).

### Arbuscular mycorrhizal fungi

The phylum Glomeromycota (i.e. AMF) is divided in four orders, with most described species belonging to the Glomerales and Diversisporales (Schüßler *et al.*, 2001b). *Glomus* is the largest genus in the phylum, with > 70 morphospecies (Redecker & Raab, 2006), with *Glomus* group A accounting for much of this diversity. *Glomus* dominates AMF communities in many field settings, where 70% of the AMF have been identified as *Glomus* (range 60–85%) (Helgason *et al.*, 1998; Vandenkoornhuysen *et al.*, 2002; Zhaoyong *et al.*, 2006; Alguacil *et al.*, 2009; Öpik *et al.*, 2009, 2010; Sonjak *et al.*, 2009; Wilde *et al.*, 2009), and shows the highest root colonization rates among the Glomeromycota taxa (Hart & Reader, 2002). There is some controversy on as to whether AMFs have dispersal limitation (Lekberg *et al.*, 2007; Dumbrell *et al.*, 2010) or whether they can disperse at the scale of km, recording different dispersal vectors such as animals (Janos & Sahley, 1995; Mangan & Adler, 2000; Lekberg *et al.*, 2011), wind (Warner *et al.*, 1987) and land movements associated with agriculture (Rosendahl *et al.*, 2009). *Glomus* species are the most common taxa recorded in these dispersal studies.

### Root sampling

We performed a plant sampling scheme aimed at including most of the plant species in the community and reflecting the relative abundance of each species sampled. A total of 37 vegetation clumps, with one to eight plant species (average 2.7), were sampled along two transects of 500 m<sup>2</sup> each. A total of 130 individuals of 37 plant species, representing 66% of all the species in the community, were sampled (see species in Table 1). Rarely, a vegetation clump had more than one individual of the same species; in those cases only one of the individuals was sampled. We have considered relative abundance as an intrinsic characteristic of each plant species in a given community that can influence its interaction pattern with other species. Accordingly plant–AMF interaction matrices should be built from surveys that reflect the relative abundance of each species. The root tips were unearthed, cut and dried with silica gel for further DNA extraction.

### DNA extraction

The youngest tips of the nonlignified roots were selected from plant samples, as they often show a higher proportion of Glomeromycota colonization. Root tips were cut and placed in 2 ml Eppendorf tubes with 2.3 mm stainless steel beads. Then root tissues were pulverized in a Retch MM400 (Biometra, Madrid, Spain) tissue lyser.

**Table 1** Number of individuals sampled for each plant species

Plant species	Positive	Negative	Total
<i>Neobuxbaumia tetetzo</i>	18	0	18
<i>Mimosa luisana</i>	15	1	16
<i>Mammillaria colina</i>	8	4	12
<i>Coryphantha pallida</i>	7	0	7
<i>Ruellia hirsuto-glandulosa</i>	6	0	6
<i>Siphonoglossa ramosa</i>	4	1	5
<i>Agave macroacantha</i>	3	1	4
<i>Caesalpinia melanadenia</i>	3	4	7
<i>Calliandra eryophylla</i>	3	0	3
<i>Acacia constricta</i>	2	0	2
<i>Cardiospermum halicacabum</i>	2	0	2
<i>Dalea</i> sp.	2	2	4
<i>Justicia mexicana</i>	2	0	2
<i>Mammillaria carnea</i>	2	1	3
<i>Mammillaria casoi</i>	2	0	2
<i>Mascagnia seleriana</i>	2	0	2
<i>Sanvitalia fruticosa</i>	2	0	2
<i>Viguiera dentata</i>	2	0	2
<i>Allionia incarnata</i>	1	2	3
<i>Agave karwinskii</i>	1	0	1
<i>Bouteloua gracilis</i>	1	0	1
<i>Bursera aloexylon</i>	1	3	4
<i>Cathestecum brevifolium</i>	1	0	1
<i>Ditaxis guatemalensis</i>	1	0	1
<i>Echynopterix eglandulosa</i>	1	0	1
<i>Eysenhardtia polystachya</i>	2	1	3
<i>Ferocactus latispinus</i>	1	0	1
<i>Hemiphylacus latifolius</i>	1	1	2
<i>Ipomoea</i> sp.	1	0	1
<i>Lantana achyranthifolia</i>	1	0	1
<i>Lantana camara</i>	1	1	2
<i>Loeselia caerulea</i>	1	0	1
<i>Senna wislizenii</i>	1	2	3
<i>Solanum trydinamum</i>	1	0	1
<i>Thompsonella minutiflora</i>	1	1	2
<i>Mammillaria haageana</i>	0	1	1
<i>Jatropha neopauciflora</i>	0	1	1
Total	103	27	130

Plant species are ranked by their abundance in positive amplification and sequencing of *Glomus* group A.

Total DNA was extracted using the DNeasy Plant Minikit (Qiagen, Las Matas, Madrid, Spain) with the addition of 0.33% final concentration of PVP40 to buffer AP1, which facilitated the elimination of some PCR inhibitor compounds, and we then subsequently followed the manufacturer's instructions. As these extracts contained a mixture of DNA from fungi and the host plant, DNA quantification was routinely omitted and crude extracts were used for subsequent PCRs.

### Glomeromycota internal transcribed spacer (ITS) amplification and sequencing

A nested PCR protocol was used for the amplification of the samples. Primary PCR amplified the whole ITS region, including ITS-1, 5.8S and ITS-2. This was conducted in 25  $\mu$ l volume including 1  $\times$  *Taq* Buffer (Biotools, Madrid, Spain), 3 mM MgCl<sub>2</sub>, 0.5 mM each of dNTP, 0.4 mg ml<sup>-1</sup> BSA, 12.5 pmol

each of NS5 (forward) and ITS4 (reverse) primers of White *et al.* (1990), 1 U of *Taq* DNA polymerase and 1 µl of crude DNA extract. The PCR program consisted of an initial DNA melting step of 3 min at 95°C followed by 30 cycles each of 30 s at 95°C, 30 s at 51°C for annealing and 2 min at 72°C for extension. After a final extension step of 10 min at 72°C, PCRs were kept at 4°C. One microlitre of this PCR was used as template for the nested PCR. Four primer-pair combinations were assayed for the nested PCR in an attempt to detect as much diversity as possible among Glomeromycota. The PCR cocktail was identical to that of the primary PCR except for the primer-pair used, which included Forward/Reverse, Glom1310/ITS4i (Redecker, 2000; Redecker *et al.*, 2003), for the amplification of *Glomus* group A (Schüßler *et al.*, 2001a); LETC1670/ITS4i (Redecker, 2000) for the amplification of *Glomus* group B (Schüßler *et al.*, 2001a); NS5/GIGA5.8R (Redecker, 2000) for the amplification of Gigasporaceae; and ACAU1660/ITS4i (Redecker, 2000) for the amplification of Acaulosporaceae. The PCR program consisted of an initial DNA melting step of 3 min at 95°C followed by 30 cycles each of 45 s at 95°C, 50 s at 56°C for annealing and 1.5 min at 72°C for extension. After a final extension step of 10 min at 72°C, PCRs were kept at 4°C. PCR products were checked on 1% agarose gels. PCR protocols were optimized for two of these groups of AMFs with available axenic cultures of *Glomus* group A and *Gigaspora* sp. Of these four primer-pair combinations, no amplification was obtained for the families Gigasporaceae and Acaulosporaceae. Less than 30% amplification success was obtained for *Glomus* group B primer-pair, whereas for the primer-pair of *Glomus* group A, 78.21% success was achieved, suggesting a predominance of this group of *Glomus* in the AMF communities in the study area. Subsequent sequencing of PCR products was continued only with this monophyletic group of *Glomus*.

Positive amplifications of the expected size were cloned into pGEM-T easy vector (Promega, Madrid, Spain) and transformed onto X-Gal, IPTG ampicillin, LB agar plates. Positive colonies were screened with T7 and SP6 vector primers for inserts of the appropriate size, then cultured for miniprep plasmid extraction (Roche, Penzberg, Germany) and sequenced with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing was performed by Macrogen Inc. (Seoul, Korea). Forward and reverse sequences were compared, assembled and corrected where necessary using SEQUENCHER® (GeneCodes Corp., Ann Arbor, MI, USA), thus establishing the consensus sequence of each sample. BLAST searches were performed to reliably assign sequences to AMF. BLAST searches were performed on forward, reverse and consensus individual sequences in order to detect possible chimeras (Schechter & Bruns, 2008). Only those that matched a Glomeromycota sequence in both forward and reverse sequences and rendered high bit scores (> 1300) and low *E* values in the consensus sequences were selected for the analysis. This procedure is especially suitable for pairwise comparisons of sequence from closely related species. In these cases evolutionary processes involving natural recombination and incomplete lineage sorting could be identified as false chimera positives in specific software for the detection of chimeras (Schechter & Bruns, 2008).

## DNA sequence alignment and analysis

Sequences were aligned with ClustalW (Thompson *et al.*, 1994) implemented in MEGA4 (Tamura *et al.*, 2007). Sequence alignments were corrected by visual inspection with BioEdit v. 7.0.9 (Hall, available at <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>).

Pairwise distance matrices were computed using the default values in Dist.seqs implemented in Mothur (Schloss *et al.*, 2009). These served as input for Bin.seqs in order to cluster the sequences into operational taxonomic units (OTUs) of a defined sequence identity. The OTUs were defined according to their sequence dissimilarity at different cutoff values, which spanned 1–10% of their sequence being different. This approach seems reasonable since species concepts are difficult to apply in AMFs (Redecker *et al.*, 2003). Thus, using sequence bins rather than taxonomic assignments based on BLAST analyses is more meaningful for environmental samples without prior information on AMF diversity information, because not all the sequences may match an identified sequence in the database and the use of sequence similarities prevents uncertainties associated with fungal taxonomy and classification.

We used rarefaction curves to illustrate how the number of OTUs increases with the number of sequences sampled. Rarefaction curves were performed for each cutoff. The value of OTUs levels off and reaches an asymptote when more sequences sampled do not reveal more OTUs, indicating sufficient sampling. Confidence intervals for OTUs were calculated based on 1000 randomizations.

## Network analyses

For each cutoff, plant–AMF interactions were characterized as bipartite networks consisting of two sets of nodes, plant species, in rows, and AMF OTUs, in columns. Pairs of each type of nodes were considered linked (i.e. interaction) if an AMF OTU was present in a given plant species roots. This qualitative 0/1 matrix was used to calculate network parameters to describe connectance, nestedness and modularity. Connectance is considered as the realized proportion of possible links (Yodzis, 1980), in this case, the proportion of pairs of plant–AMF OTU that directly interact. It was calculated using the bipartite package for R (Blüthgen *et al.*, 2006).

The nestedness concept describes a particular pattern of interaction in which specialists interact with species (or OTUs) that form perfect subsets of the species (or OTUs) with which generalists interact (Bascompte & Jordano, 2007). Nestedness parameters measure how the presence/absence pattern of interactions departs from the perfect nestedness. We used two of the most common metrics to estimate nestedness: temperature index (Atmar & Patterson, 1993) and nested overlap and decreasing fill (NODF) (Almeida-Neto *et al.*, 2008). The significance of nestedness was assessed by comparing the observed nestedness with the frequency distribution of that metric calculated using 1000 replicates of null model II (Bascompte *et al.*, 2003). Null model II uses equal dimension matrices in which each cell of the interaction matrix has a given probability of

being occupied. This probability is the arithmetic mean of the connection probability of the focal plant species and AMF OTU. Accordingly, deviations from this null model result solely from an asymmetric distribution of interactions between species (Vacher *et al.*, 2008).

Nested overlap and decreasing fill values are matrix dimension-dependent and accordingly they are unsuitable to compare across studies. In order to allow cross-network comparisons with other mutualistic systems, the relative nestedness was calculated. This measure corrects for variation in species and OTU richness and also in the number of links. Relative NODF is defined as  $(\text{NODF}_{\text{observed}} - \text{NODF}_{\text{null model}}) / \text{NODF}_{\text{null model}}$ , where  $\text{NODF}_{\text{observed}}$  is the nestedness of the actual matrix, and  $\text{NODF}_{\text{null model}}$  is the average nestedness of random replicates generated from the null model.

Nested overlap and decreasing fill was also calculated independently for rows (plant species) and columns (AMF OTUs) and the statistical significance assessed by comparing against the null model II. Nestedness metrics are influenced by order of rows and columns in the matrix. In order to make our results comparable with previous studies, rows and columns were ordered by interaction abundance before the calculation of nestedness metrics. Nestedness metrics were calculated with the help of the software ANINHADO (Guimarães & Guimarães, 2006).

Modularity reflects the fact that there are groups of species that tend to interact more within species in the same group than is expected by chance. Nodes of a network can be grouped into modules, in such a manner that the number of links within the modules is maximized and the number between modules is minimized. A simulated annealing optimization approach was used to detect modules that maximized modularity (i.e. proportion of links within vs between modules) (Guimerà & Amaral, 2005a,b). Because of its heuristic nature, 10 runs of the algorithm were conducted for each cutoff, but the variation in modularity was negligible (SE of the modularity across the 10 runs ranged from 0.0190 to 0.0192 across the different cutoffs). We report the maximum value of modularity obtained in the 10 runs. Although our network is bipartite, we used a modularity algorithm for unipartite networks. Because our plant–AMF network is a two-party network, one could conclude that an algorithm for a two-party network (i.e. bipartite) would be more appropriate. However, this decision depends on the question addressed. For example, algorithms for modularity in a bipartite network search for independent groups of plants (or AMFs) that share a similar interaction pattern (i.e. that interact with the same AMFs (or plants)); whereas algorithms searching for modularity in a unipartite network identify mixed groups of plants and AMFs tightly inter-related (see Olesen *et al.*, 2007 for a more detailed explanation). As we are interested in the groups of plants and AMFs that are highly connected to each other, rather than in groups of plants and/or AMFs created as a function of their shared interactions, we used algorithms searching for modularity in a unipartite network (see Fortuna *et al.*, 2010, for the appropriateness of this method). However, we also tested for modularity using a bipartite network and the results consistently showed that our networks were composed of modules usually grouping the same species grouped

by the modules in the unipartite approach (see Olesen *et al.*, 2007 for a similar comparison). Only modularity for a unipartite network is reported. Modularity significance was tested by comparing it to the null case of modularity calculated using 100 random graphs with the species ranked according to their degree distribution in the original network (Guimerà *et al.*, 2004). Modularity was calculated and its significance tested using the software Netcarto (Guimerà *et al.*, 2004; Guimerà & Amaral, 2005a,b).

In order to provide a generalization to our results, we compared them with other studies in which plant–AMF interactions had been intensively surveyed at the community level. After inspecting the 138 studies cited in the MaarjAM database (Öpik *et al.*, 2010) and relevant references within, we found only two studies which aim to survey most of the plant species in the community: Davison *et al.* (2011) and Öpik *et al.* (2009). These studies are not independent as they share data from the same site. In these studies 10–11 plant species were used and 40–51 AMF OTUs were recorded, belonging to Glomeraceae, Gigasporaceae, Acaulosporaceae and Diversisporaceae. We reanalysed their data calculating the same nestedness and modularity estimates as described for this study.

Although the qualitative analyses described only consider the presence or absence of an interaction, a species number of interactions can be highly influenced by its abundance, which might have an effect on nestedness (Vázquez, 2005) and modularity. In the next section we use biological information contained in our data to estimate the relative contribution of plant species abundance on plant–AMF interaction pattern.

#### Relative contribution of plant abundance to plant species number of AMF interactions

A plant species with high relative abundance in the community will have a higher probability of interacting with a higher number of AMFs, because more individuals will be sampled of this plant species. However, a plant species' tendency to interact with a given number of AMF OTUs can also result from other processes independent of its relative abundance. Other biological processes, such as habitat heterogeneity, demographic dynamics, plant–AMF overlapped phenology, AMF competition within the root or specific selectivity in plant–AMF associations, can also produce a nonrandom pattern of plant–AMF interactions independent of the species' relative abundance. While abundance-dependent interaction patterns occur at the species level as an effect of adding up multiple individuals, interaction patterns resulting from abundance-independent processes should be observed at the individual level.

Accordingly, we have calculated plant–AMF interactions at the individual plant level (AMF load) in order to characterize these biological processes independent of species abundance. We define AMF load as the plant species' mean number of AMF OTUs per individual. Several tangled processes can be underlying a given species AMF load, and further experiments can be designed to elucidate these processes. Although we cannot tease apart the specific mechanisms resulting in a given AMF load, this index is

independent of plant species' relative abundance considering that AMF load is calculated as an average of the individuals' trait within a plant species.

We tested if there is a statistically significant relationship between relative plant abundance and AMF load on the number of plant species links in the network, using a generalized linear model with a Poisson distribution of errors. Plant species degree in the network (number of links per plant species) was used as the dependent variable and the number of individuals sampled and AMF load per plant species were used as independent variables. The relative contribution of plant relative abundance and AMF load to explain the variance in the number of plant species links in the network was estimated as the ratio of the standard deviations of the two effects, as implemented in the *relimp* package for R (Silber *et al.*, 1995).

## Results

### AMF OTU definition and rarefaction curves

Positive amplification for *Glomus* group A was detected in 103 out of the 130 plants sampled (79.23%) (Table 1). Positive amplification was obtained from at least one of the individuals sampled for each species, except for *Mammillaria haageana* and *Jatropha neopauciflora* (Table 1).

A total of 95 out of the 1909 sequenced clones (4.98%) produced unreadable sequences, 251 (13.15%) corresponded to other coamplified fungi, 40 (2.10%) to chimeric sequences, and 1523 (79.77%) to Glomeromycota with BLAST scores above 1300. Further analyses were based on this subset of 1523 sequences (Genbank numbers JN194215 to JN195737).

The number of different AMF OTUs varied depending on the predefined cutoff values of genetic dissimilarity (Supporting Information, Fig. S1). Rarefaction curves showed that for the cutoff which grouped together sequences with a genetic difference smaller than 1% (Fig. S1a), 163 AMF OTUs were identified out of 1523 sequences. Rarefaction curves did not reach the stabilization until the cutoff value of 5% (Fig. S1e) and more strictly at 8% (Fig. S1h). For the 5% and 8% cutoffs, 34 and 23 different OTUs were identified, respectively. The cutoff which grouped together sequences with genetic difference smaller than 10% identified 14 AMF OTUs out of 1523 sequences (Fig. S1j).

### Plant–AMF networks

The number of plant species in the network was 35 and the number of AMF OTUs ranged from 163 to 14 depending on the cutoff value considered (Table 2). Ten networks (i.e. one network per cutoff) were built grouping within an OTU all the sequences in 10 increments of 1% in dissimilarity intervals (i.e. 1–10%). Approximately 11% of the possible interactions between plant species and AMF OTUs were actually realized (average connectance across different cutoffs (mean  $\pm$  SE: 11.5  $\pm$  0.01) (Fig. 1; see Figs S2–S10 for all the other cutoffs).

All 10 networks were significantly more nested than expected by chance, both considering the overall network and considering rows and columns independently (for all cases  $P < 0.001$ , Table 2). The degree of nestedness was independent of the cutoff considered (Pearson  $R^2 = 0.57$ ,  $n = 10$ ,  $P > 0.05$ ). NODF of the overall network ranged from 14.36 to 54.83, corresponding, in all cases, to a temperature  $> 92$  (Table 2).

When plant species and AMF OTUs are analysed independently (i.e. rows and columns), both showed high NODF values (Table 2) indicating that there is a tendency of specialists to interact with generalists. However, plant species had a relative nestedness more than twofold higher than the AMF OTU nestedness (Table 2), indicating that this pattern is stronger for plants than for AMFs.

All networks except for the cutoff of 10% show a significant modularity (Table 2). For the 10% cutoff, the number of species in the network was 49 (35 plant species and 14 AMF OTUs). According to Olesen *et al.* (2007), when networks are based on  $< 50$  species, it is probably the detection of just one single module. Thus, for all the cutoffs in which modularity was detectable, we found significant values of modularity. The number of modules varies from five to nine across the different cutoffs and the average number of nodes within a module range from nine to 22 (Table 2). Plant and AMF species ascribed to different modules coexist in the same vegetation clump, with an average of 2.6 (range one to five) modules per vegetation clump.

In this study we only found AMFs belonging to Glomeraceae, but the observed pattern of plant–AMF interaction might vary in other systems with higher AMF phylogenetic diversity. In order to explore the variability of the observed network pattern across other AMF phylogenetic diversity scenarios, we compared our results with two other studies in which other less abundant families of AMFs have been recorded (i.e. Gigasporaceae, Acaulosporaceae, Diversisporaceae). Both networks presented in Öpik *et al.* (2009) and Davison *et al.* (2011) show significant nestedness in the overall network, and also for plants and AMFs independently (relative  $\text{NODF}_t = 0.25\text{--}0.28$ ;  $\text{NODF}_p = 0.50\text{--}0.54$ ;  $\text{NODF}_{\text{AMF}} = 0.24\text{--}0.26$ ; all  $P < 0.05$ ;  $t$ , total;  $p$ , plants, AMF, arbuscular mycorrhizal fungi) (Fig. S11). In addition, in these two networks, the nestedness for plants was also stronger than nestedness for AMFs. Accordingly, regarding nestedness, our results, which only detected one group of the most abundant AMFs, were in agreement with their results, which recorded a broader AMF phylogenetic diversity. However, the connectance in those two networks was higher than in our study (42–41%) and no significant modularity was observed.

### Relative contribution of plant abundance to plant species number of AMF interactions

The mean number of plant individuals per species ranged from one to 18 (mean = 2.9; SD = 3.8). Plant species have, on average, an AMF load of 3.6 AMF OTUs per individual (SD = 1.5; range = 1–7) considering the cutoff of 1%, and an average of 1.3 (SD = 0.5; range = 1–3) considering the cutoff of 10%, with the estimates for the rest of the cutoffs contained within the values

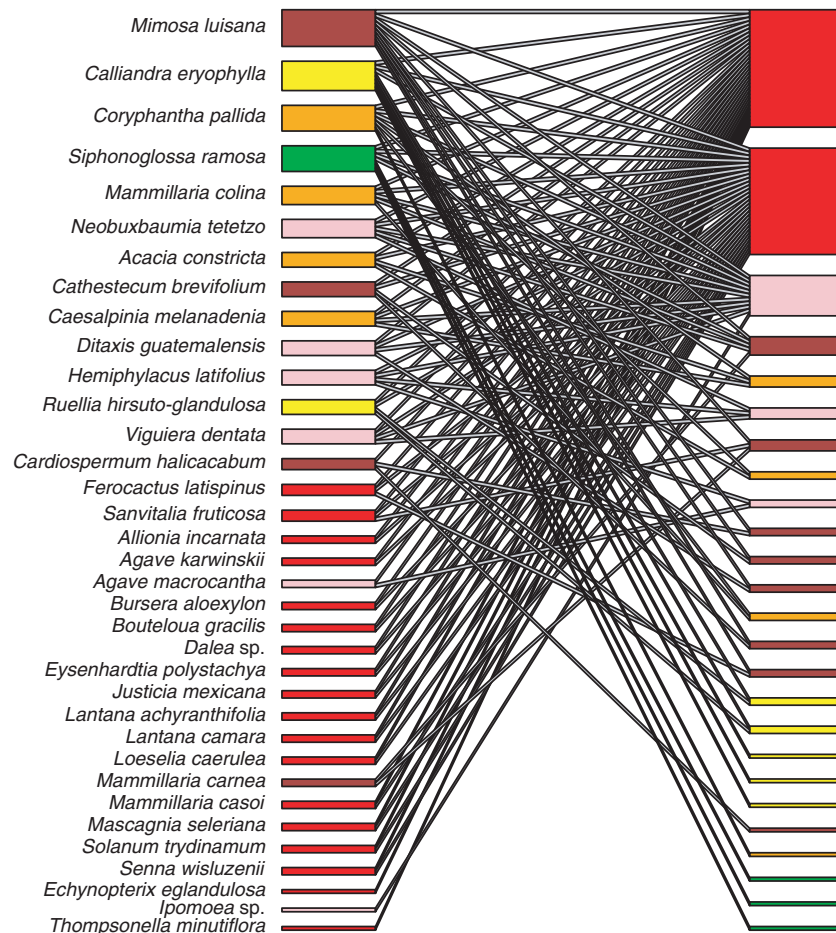
**Table 2** Descriptive statistics of the plant–mycorrhizal fungi network

Cutoff %	N	I	C	T	Nestedness							Modularity		
					NODF <sub>t</sub>	NODF <sub>p</sub>	NODF <sub>AMF</sub>	Relative			Modules	Nodes	Modularity	
								T	NODF <sub>t</sub>	NODF <sub>p</sub>				NODF <sub>AMF</sub>
1	163	313	5.5	95	14.4	20.6	14.1	15	0.6	1.4	0.6	9	22 (3–35)	0.57
2	85	232	7.8	94	22.4	31.0	20.9	18	0.8	1.3	0.7	7	17 (7–22)	0.48
3	61	194	9.1	95	28.3	44.1	23.2	20	0.9	1.6	0.6	8	12 (6–18)	0.44
4	45	168	10.1	94	35.0	49.9	26.0	21	0.9	1.5	0.6	6	13 (5–21)	0.42
5	34	146	12.3	92	42.0	55.2	27.9	21	1.0	1.3	0.5	8	9 (4–15)	0.39
6	30	132	12.6	92	46.8	60.4	28.3	21	1.0	1.3	0.5	7	9 (4–14)	0.38
7	25	114	13.0	93	47.9	58.6	26.7	22	0.9	1.1	0.4	6	10 (4–20)	0.40
8	23	111	13.8	93	49.6	58.4	28.9	22	0.9	1.0	0.4	6	10 (7–20)	0.30
9	19	105	15.8	93	52.0	57.4	33.2	26	0.8	0.9	0.4	6	9 (6–19)	0.37
10	14	70	14.3	96	54.8	58.5	30.8	27	1.1	1.1	0.5	5	10 (4–21)	0.42 <sup>ns</sup>

Cutoff %, percentage of dissimilarity used as cutoff; N, number of operational taxonomic units (OTUs) of *Glomus*; I, number of interactions; C, connectance:  $(100 \times I)/(N \times 35)$ ; T, nestedness calculated as matrix temperature; NODF<sub>t</sub>, nested overlap and decreasing fill for the overall matrix; NODF<sub>p</sub>, NODF for plants; NODF<sub>AMF</sub>, NODF for *Arbuscular mycorrhizal fungi*; Relative T,  $(T - T_{\text{null model}})/T_{\text{null model}}$  (see the Materials and Methods section for details about the null model); NODF<sub>t,p,AMF</sub>,  $(\text{NODF}_{t,p,AMF} - \text{NODF}_{\text{null model}})/\text{NODF}_{\text{null model}}$ .

Modules, number of modules; nodes, mean number of nodes per module (range) and modularity values.

For all nestedness and modularity parameters,  $P < 0.05$  except where 'ns' is shown. Number of plant species in every cutoff is 35.



**Fig. 1** Bipartite network showing the interactions between plants (left) and arbuscular mycorrhizal fungi (AMF) operational taxonomic units (right) obtained with the 7% cutoff. Species are ordered from generalist (top) to specialist (bottom) and colours represent nodes included in the same module, with modules representing subsets of species more tightly interconnected.

presented. *Dixitalis guatemalensis* and *Cathestecum brevifolium* were consistently the species that had higher AMF load across cutoffs, and *Thompsonella minutiflora*, *Echynopterix eglandulosa* and *Mammillaria casoi* were the species with the lowest AMF load. Both plant species abundance and AMF load have a significant effect on plant species degree, for every cutoff considered (Table 3). The relative contribution of plant relative abundance and AMF load to plant species degree is similar for cutoffs of 4–10% (Table 3). Only in the lower cutoffs, such as the 1%, can plant relative abundance explain 1.7 times more variation in plant species degree than AMF load (Table 3).

## Discussion

In this paper we present the network properties of a plant–AMF mutualistic system. Our results show a nonrandom interaction pattern in plant–AMF associations with a network with low connectance, highly nested and modular. As expected, the nestedness values observed are concordant with other mutualistic networks (Bascompte & Jordano, 2007) and the modularity detected reinforces the hypothesis that selectivity in plant–AMF interactions can result in emergent patterns at the community level.

Our gradient of cutoffs (1–10%) adequately characterize both intraspecific and interspecific variation for Glomeromycota. The average of intraspecific ITS variability in this taxon is 7.46% (SD 4.14), with some examples of intraspecific variation of 8.7% and 5.9% variability in *Glomus intraradices* and in *Glomus mosseae* (Nilsson *et al.*, 2008). The stabilization of rarefaction curves between cutoffs close to intraspecific variation of the taxa (5%, or more strictly 8%) indicates that our sampling captured a considerable amount of the total diversity of the *Glomus* A group of AMFs present in the area. Finding consistent network properties across cutoffs supports the idea that the network structure is maintained independently of the genetic differentiation threshold considered to define AMF OTUs. Regarding comparisons with other studies reanalysed in this paper, the presence of nestedness seems to be a consistent pattern, although there is high variability

in its strength across different plant–AMF communities. Modularity in plant–AMF networks seems to be a less consistent pattern across sites and potentially influenced by the degree of connectivity in each community. Interestingly, although both plants and AMF show a nested structure, plants have a stronger pattern of nestedness than AMF. We discuss these results in turn in the following.

In general, mutualistic networks are characterized by having low connectance and being highly nested (Bascompte & Jordano 2007). Our plant–AMF network showed similar connectance to that reported for pollination networks ( $11.89 \pm 3.41$ ; Olesen *et al.*, 2006) and plant-to-plant facilitation networks ( $24.9 \pm 2.68$ ; Verdú & Valiente-Banuet, 2008), but other plant–AMF networks show higher connectance (Öpik *et al.*, 2009; Davison *et al.*, 2011). Regarding nestedness, our plant–AMF network showed similar, high values of nestedness ( $T$ ) than the ones reported for other positive interactions such as plant-to-plant facilitation ( $89.7 \pm 2.7$ ; Verdú & Valiente-Banuet, 2008), seed dispersal ( $84.3 \pm 2.1$ ) and pollination networks ( $85.3 \pm 2.2$ ; Bascompte *et al.*, 2003). A pattern of generalist plants tending to associate with generalist AMFs, has previously been reported for plant–AMF systems (Davison *et al.*, 2011). Our reanalysis of this and another related study (Öpik *et al.*, 2009) does indeed show significant nestedness, suggesting that this may be a general pattern in plant–AMF networks.

Ecological networks with low connectance and highly nested have a tendency to be highly modular (Fortuna *et al.*, 2010). This is also the case of the network presented in this study. However, the other two published networks analysed were nested but not modular. Interestingly, these studies have high connectance and, according to the pattern revealed by Fortuna *et al.* (2010), highly connected networks tend to be either nested or modular, but not both. Further studies looking at plant–AMF community interactions are needed to elucidate a general pattern regarding the degree of connectance and its influence on modularity in different communities.

The nonrandom pattern of plant–AMF interactions observed at the community level can be produced by diverse mechanisms.

**Table 3** Generalized linear models (GLM) testing for the effects of plant species' relative abundance and arbuscular mycorrhizal fungal (AMF) load on the species degree in the network

Cutoff %	$R^2$	Plant abundance	Individual AMF load	Relative importance of plant abundance/individual AMF
1	0.87	0.14 (0.01)***	0.22 (0.04)***	1.7***
2	0.84	0.12 (0.01)***	0.25 (0.05)***	1.4***
3	0.79	0.12 (0.01)***	0.28 (0.06)***	1.3***
4	0.74	0.11 (0.01)***	0.28 (0.06)***	1.1***
5	0.77	0.09 (0.01)***	0.28 (0.07)***	1.1***
6	0.64	0.09 (0.01)***	0.34 (0.11)**	1.2***
7	0.63	0.08 (0.02)***	0.37 (0.11)**	1.1***
8	0.63	0.08 (0.02)***	0.38 (0.11)***	0.96***
9	0.85	0.07 (0.02)***	0.35 (0.12)**	1.05***
10	0.64	0.08 (0.02)***	0.57 (0.19)**	1.08***

Cutoff %, percentage of genetic sequence dissimilarity;  $R^2$ , pseudo- $R^2$  of the full model.

Plant abundance and individual AMF load effect, mean (SE) and  $P$ -value of each effect, respectively; relative importance of plant abundance/individual AMF, ratio of the standard deviations of the two effects on plant species degree.

\*,  $P < 0.05$ ; \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.001$ .



Ecological processes such as habitat heterogeneity, specific selectivity in plant–AMF associations, plant–AMF overlapped phenology, species' relative abundance and phylogenetic diversity or AMF competition within the root can produce modularity and nestedness in plant–AMF networks.

Modularity can emerge from processes such as habitat heterogeneity, resulting in nonrandom species spatial distribution (Olesen *et al.*, 2007). However, in this system, species from different modules coexist in the same vegetation clumps, suggesting that species distribution does not constrain plant–AMF interaction patterns. The modularity found at the community level is consistent with previous studies which have confirmed both qualitative (Ravnskov & Jakobsen, 1995) and quantitative (Bever *et al.*, 1996; Streitwolf-Engel *et al.*, 1997; Eom *et al.*, 2000) selectivity in AMF and plant interactions.

In our study we have detected only AMFs belonging to the Glomeraceae. However, other orders may be present in the area in a different season or in different habitats such as soil (spores) vs roots (Camargo-Ricalde *et al.*, 2003). In a more phylogenetically diverse AMF community, new interactions will be found, which might affect the network structure. However, our results show that in other communities with higher AMF phylogenetic diversity, nestedness is maintained. This is because the AMF taxa missing in our study, Gigasporaceae, Acaulosporaceae and Diversisporaceae, tend to interact with generalist plant species (Fig. S11). However, because our results are based on very few communities, further studies are required to confirm that the observed pattern can be generalized to overall plant–AMF associations.

Relative abundance of plant species can also lead to a nonrandom interaction pattern, as AMF OTUs with few interactions will have a higher probability of interacting with abundant plant species than with scarce ones. Our results show that, although both plants and AMFs present a nested structure, plants have a stronger pattern of nestedness than AMFs. A potential explanation for this difference might be that sampling is different for plants and AMFs. While we can observe plants and account for their relative abundance in our survey, AMF sampling has followed a blind procedure, and it does not necessarily represent relative abundances precisely. Our data support the fact that species' relative abundance contributes significantly to the network structure; however, it has been shown that species abundance cannot fully explain the observed interaction pattern, and its relative importance is similar to other ecological processes.

An alternative explanation for differences in plant and AMF nestedness is that mutualistic networks can result in a lack of nestedness because of a balance between mutualism and competition. In this particular situation, competition can force generalist species to become more specialist (Ricciardi *et al.*, 2010). A combination of mutualism and competition might well be happening in plant–AMF systems (Husband *et al.*, 2002). However, while AMF compete for root space with other AMF when they interact with a generalist plant species (i.e. with a high AMF load), this is not true for the case of a plant interacting with a generalist AMF taxa.

The overall network nestedness suggested a higher importance of mutualism over competition in AMF and plant community

interactions. These mycorrhizal networks established among plants inhabiting multispecific vegetation clumps could alleviate competition among neighbouring plants, promoting plant-to-plant facilitation (Castillo *et al.*, 2010; Verdú & Valiente-Banuet, 2011). Plant species inhabiting a more diverse phylogenetic neighbourhood can benefit from a higher AMF diversity in their rhizosphere (Maherali & Klironomos, 2007). This pattern might help to explain the phylogenetic overdispersion found in plant communities from environments where facilitation is a key process establishing community assemblage (Verdú & Valiente-Banuet, 2008). In support of this idea, nestedness and modularity have been shown to be influenced by species phylogeny (Valiente-Banuet & Verdú, 2007; Rezende *et al.*, 2009; Verdú *et al.*, 2010; Verdú & Valiente-Banuet, 2011) and complementary traits (Rezende *et al.*, 2009) in other systems.

In conclusion, nonrandom patterns emerge from analysing plant–AMF interactions at the community level consistent with previous knowledge. Provided that plant–AMF interactions are organized in certain groups of species within which interactions are more frequent, new biological questions are generated by our results. Do species within a module share certain traits? Do plant and fungi phylogenies explain the observed interaction pattern? Closely related species might tend to interact with the same partners, resulting in closely related species sharing membership of a network module. Answering these questions will increase our understanding of the largely unknown potential influence of plant–AMF coevolutionary history in plant community assemblages.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Rarefaction curves for the Glom1310/ITS4i ITS sequences at increasing dissimilarity levels ranging from 1 to 10%.

**Figs S2–S10** Bipartite networks for each cutoff (1–10% genetic differentiation, except for the 7% cutoff shown in the main text).

**Fig. S11** Interaction matrices of our data (cutoff 7%), Davison *et al.* (2011), and Öpik *et al.* (2009).

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