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Spatial Structure and Interspecific Cooperation: Theory and an Empirical Test Using the Mycorrhizal Mutualism

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ABSTRACT: Explaining mutualistic cooperation between species remains a major challenge for evolutionary biology. Why cooperate if defection potentially reaps greater benefits? It is commonly assumed that spatial structure (limited dispersal) aligns the interests of mutualistic partners. But does spatial structure consistently promote cooperation? Here, we formally model the role of spatial structure in maintaining mutualism. We show theoretically that spatial structure can actually disfavor cooperation by limiting the suite of potential partners. The effect of spatial structuring depends on the scale (fine or coarse level) at which hosts reward their partners. We then test our predictions by using molecular methods to track the abundance of competing, closely related, cooperative, and less cooperative arbuscular mycorrhizal (AM) fungal symbionts on host roots over multiple generations. We find that when spatial structure is reduced by mixing soil, the relative success of the more cooperative AM fungal species increases. This challenges previous suggestions that high spatial structuring is critical for stabilizing cooperation in the mycorrhizal mutualism. More generally, our results show, both theoretically and empirically, that contrary to expectations, spatial structuring can select against cooperation.

Keywords: arbuscular mycorrhizal fungi, plant benefit, cheaters, tillage, sanctions, partner choice.

Introduction

Despite the ubiquity of cooperative mutualisms between species, explaining their evolutionary persistence remains a major challenge (Sachs et al. 2004; West et al. 2007). The problem is that mutualisms are vulnerable to “free-riders” or “cheaters,” partners who do not cooperate but may gain the benefit of others cooperating (Axelrod and Ham-

ilton 1981). These exploiters are common in mutualistic systems, ranging from rhizobia that fail to provide fixed N₂ to their legume hosts (Denison and Kiers 2011) to pollinators that lay eggs in hosts but defect from pollinating duties (Goto et al. 2010). Theory suggests that, without mechanisms to prevent exploitation, natural selection should favor these free-riders because they pay reduced costs but still reap benefits of the partnership.

The spatial structuring or partitioning of symbiotic partners is often invoked as a mechanism for maintaining mutualisms (Frank 1994; Doebeli and Knowlton 1998; Bever and Simms 2000; Hoeksema and Kummel 2003; Foster and Wenseleers 2006; Gardner et al. 2007; Lion and van Baalen 2008; Platt and Bever 2009; Hodge et al. 2010). In particular, spatial structuring has been proposed to be important in stabilizing mutualisms in which hosts differentially allocate resources to partners varying in benefit. It has been argued that spatial structure can facilitate the evolution of cooperation by separating patches of high-quality mutualists from their antagonistic counterparts on a single host (Bever et al. 2009). When mutualists of similar quality are clustered, a host may preferentially reward patches of cooperators and/or punish patches of cheaters. However, spatial structure could also limit the local diversity of partners, reducing the range of partners among which hosts might discriminate.

Here, we model the role of spatial structure in maintaining mutualism (interspecific cooperation), focusing on the symbiosis between the majority of land plants and arbuscular mycorrhizal (AM) fungi. This symbiosis, which primarily involves the exchange of carbohydrates from plants for mineral nutrients from the fungal partners, offers ideal experimental opportunities to study spatial structuring: a single host root can be colonized by several AM fungal species that range in benefit from mutualistic to

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antagonistic (Hoeksema et al. 2010). Although spatial structure has been suggested to play an important role in the maintenance of the mycorrhizal mutualism (Chanway et al. 1991; Wilkinson 1998; Bever et al. 2009; Hodge et al. 2010), observations from the field and lab experiments do not consistently reveal strong structuring of different fungal communities (van Tuinen et al. 1998; Jansa et al. 2003; Alkan et al. 2006), but rather distantly related AM fungi are found to intermingle on a small spatial scale in host roots (Jansa et al. 2003). Whether hosts discriminate among intermingled fungal partners, allocating resources preferentially to the best ones, has been the subject of an ongoing debate (Fitter 2006; Kiers and van der Heijden 2006; Bever et al. 2009; Helgason and Fitter 2009; Smith et al. 2009). Recent manipulative work supports the hypothesis that hosts reward fungal symbionts at a fine scale, preferentially allocating C to small patches of hyphae when they deliver more P resources to their host (Kiers et al. 2011). The reciprocal was also found to be true, with fungi preferentially allocating P to small patches of roots that delivered more C. This bidirectional control allows the mutualism to function like a biological market (Noë and Hammerstein 1995) in which partners offering the best rate of exchange are rewarded. However, it is not known how fungal spatial structuring influences this partner control.

To generate predictions, we first determined theoretically how the relative fitness of “high-quality” and “low-quality” mutualists should depend on the extent to which they are spatially structured. If plants can only preferentially allocate resources to fungi at a relatively coarse level, such as sections of the root system, we predict that spatial structuring will increase the relative fitness and proliferation of the high-quality mutualist. In contrast, if plants preferentially allocate resources at a much finer level, such as groups of cortical cells or at the level of the arbuscule (the site where nutrient transfer occurs; Parniske 2008), then we predict that spatial structuring will decrease the relative fitness and proliferation of the high-quality mutualist. These opposite predictions arise because spatial structure can limit the local availability of partners, reducing the extent to which hosts can reward more cooperative partners on a fine scale. We then test these competing predictions by using a multigenerational selection experiment, manipulating spatial structuring to determine how this influences the relative fitness of the low-quality AM fungal species. In the past, it has been difficult to track AM fungal fitness in mixed cultures over multiple generations. We resolved those constraints by developing specific quantitative molecular markers for our focal AM fungal species, allowing us to follow their relative fitness—when in direct competition—over multiple generations.

We manipulated spatial structure by either mixing the soil or leaving it nonmixed after every successive generation.

Predicting the Consequences of Spatial Structure

Our aim is to provide a general model that makes qualitative predictions and that could also be applied to other mutualisms, rather than a more specific model that makes quantitative predictions. We examine the situation where there is a host interacting with two mutualists (in our case, mycorrhizal fungal species) that differ in mutualistic quality. Our model defines the high-quality fungal species as a mutualist that transfers sufficient resources to the plant to be considered beneficial and the lower-quality species as one that transfers fewer resources to the plant (less beneficial/less cooperative). We assume that both fungal mutualists gain some baseline fitness benefit (w_0) by interacting with the plant, regardless of the degree of spatial structuring, but as the high-quality fungus transfers more resources, it experiences a higher energetic cost c . The fitness benefit experienced by the high-quality fungi depends upon the plant’s ability to differentiate between high- and low-quality fungi.

We assume, as has recently been shown empirically, that plants preferentially allocate resources to higher-quality mutualists (Kiers et al. 2011). We consider two evolutionary scenarios, which differ in the scale at which plants can preferentially allocate resources (coarse or fine-scale rewarding). The fungi occur in patches, where a patch is a section of the root network on a single plant. If plants can adjust their level of resource allocation only between different sections of the root network, then differential allocation is at a coarse level, between patches. In contrast, if plants are able to adjust their level of resource allocation at a finer scale, for example, toward specific groups of cortical cells or at the level of the arbuscule or interface with one fungus (Kiers et al. 2011), then differential allocation is at a fine level, within patches.

We examine the influence of population structuring by considering the extent to which fungal strains are mixed between and within root patches. At one extreme, in highly structured populations, we would expect to see each patch (associated with a single host plant) dominated by one or a small number of fungal strains but to find different strains in different patches. At the other extreme, in highly unstructured (panmictic) populations, we would expect to see a mixture of strains in each patch and a similar mix in all patches. This corresponds to Wright’s (1935) F_{st} , a measure of population structuring, going from high (1.0) to low (0). Mixing the soil would reduce the fungal population structure, resulting in a lower F_{st} . We denote the fitness effect of population structuring by $f(F_{st})$ where f increases monotonically with F_{st} and the sign of the func-

tion f differs for high- and low-quality fungal strains and is determined by the level of control the plant has over resource allocation. This function need not be the same for the two types of fungal species or for the different levels of plant control. To highlight this we use $f_{Hc}(F_{st})$, $f_{Hf}(F_{st})$, $f_{Lc}(F_{st})$, and $f_{Lf}(F_{st})$ for the high- (subscript H) and low- (subscript L) quality fungal strains under coarse (subscript c) and fine (subscript f) control, respectively.

When there is coarse control, preferential allocation by hosts is to different parts of the root network. Under this scenario, plants will be best able to discriminate between high- and low-quality fungi when each part of the root network (patch) tends to contain only one type of fungi, but different types occur in different patches. Higher population structuring (higher F_{st}) will allow greater discrimination and hence favors the high-quality fungal strain. Conversely, low-quality fungi do better if patches are more evenly mixed so the plants will be less able to discriminate against them. Therefore, fitness of a high-quality fungus (w_{Hc}) is given by $w_{Hc} = w_0 - c + f_{Hc}[F_{st}]$ and the fitness of a low-quality fungus (w_{Lc}) is given by $w_{Lc} = w_0 - f_{Lc}[F_{st}]$. This leads to the prediction that, under coarse control, the relative fitness of low-quality mutualists ($v_{Lc} = w_{Lc}/w_{Hc}$) decreases as populations become more structured (fig. 1). Conversely, we predict that spatial structuring (high F_{st} , low mixing) under coarse control favors high-quality mutualists and hence selects for cooperation.

Under fine control, we assume that the plant can allocate resources at a finer scale, toward specific fungal strains within patches of a single root-system. Consequently, plants will be best able to discriminate between low- and high-quality fungi when both types are evenly mixed (low F_{st}). Because the fitness of high-quality fungi will be positively correlated with the extent to which plants can discriminate, the fitness of a high-quality fungus when there is fine control (w_{Hf}) is given by $w_{Hf} = w_0 - c - f_{Hf}[F_{st}]$. Conversely, if patches are composed solely of low-quality partners, the plant is less able to preferentially choose the high-quality fungi, hence the fitness of a low-quality fungus (w_{Lf}) is given by $w_{Lf} = w_0 + f_{Lf}[F_{st}]$ since lower-quality mutualists will be more successful when there is low mutualist diversity (high F_{st}). These fitness equations lead to the opposite prediction from that in the coarse control scenario. When there is fine control, they predict that the relative fitness of a low-quality mutualist ($v_{Lf} = w_{Lf}/w_{Hf}$) increases as populations become more structured (fig. 1). Here, spatial structuring favors low-quality fungi and selects against cooperation. Although the extent to which this is the case, would be reduced if plants can also move resources between patches (i.e., a combination of fine and coarse control).

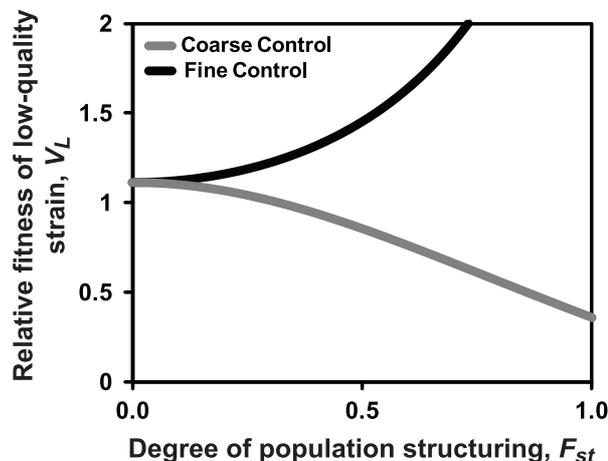


Figure 1: The effect of spatial structure (F_{st}) on the relative fitness of low-quality (less cooperative) fungal species (v_L). Term F_{st} is a measure of population structuring; high structuring ($F_{st} \rightarrow 1$) implies no mixing; low structuring ($F_{st} \rightarrow 0$) implies mixing. The black line represents coarse level control (v_{Lc} , which assumes a single plant interacts with patches of fungi on its root system), and the gray line represents fine level control (v_{Lf} , which assumes plant interacts with individual strains of fungi at the level of the arbuscule). Fine control predicts that high spatial structuring (no mixing, $F_{st} \rightarrow 1$) favors low-quality strains whereas coarse control predicts high spatial structuring (no mixing, $F_{st} \rightarrow 1$) favors high-quality strains. Here, we illustrate the simplest case, where the function f is the same for high- and low-quality fungi and for coarse and fine control, but this need not be the case. We assume the fitness function f of F_{st} takes the form $f = x(\pm F_{st})^\gamma + z$. Figure 1 is plotted for $w_0 = 1$, $x = 0.5$, $\gamma = 2$, $z = 0$ and the cost to the high-quality fungus, $c = 0.1$. Qualitatively, the result that the low-quality mutualist is favored at high F_{st} under fine control and disfavored under coarse control holds for all values of x , y , z , and $c > 0$.

Material and Methods

Culture of Plants and Fungi

For our experimental test, we used two closely related AM fungi (AMF) of the highly cosmopolitan subgenus *Glomus* Ab (Schwarzott et al. 2001), *Glomus intraradices* Schenck & Smith (but see Stockinger et al. 2009 for discussion of *G. intraradices* reclassification) and *Glomus custos* Cano & Dalpé. The use of closely related AMF allowed us to focus only on fungal cooperative strategy while excluding differences associated with radically contrasting life-history traits (Denison and Kiers 2011). Both strains were isolated from southwest Spain but represent two ends of the mutualistic continuum. At comparable colonization levels, inoculation with the “high-quality mutualist” *G. intraradices* results in significantly higher host biomass production and phosphorus (P) content compared to the “low-quality mutualist” *G. custos* in both monocot and dicot plant species (Kiers et al.

2011). We chose *Plantago lanceolata* L. as a host because it is a model plant species in AM fungal research and is readily colonized and highly responsive to a broad range of AM fungal taxa (Maherali and Klironomos 2007).

Experimental Setup

Our experiment consisted of three fungal treatments: *G. intraradices* alone, *G. custos* alone, and an equal mixture of both (“combined”). We planted seedlings in 2.6-L pots, grown from seeds that were surface sterilized with 10% sodium hypochlorite. We inoculated roots of seedlings with ~1,500 spores and 1.0 g of in vitro root material of either *G. intraradices* or *G. custos* (provided by Mycovitro Biotechnología ecológica, Granada). These cultures were originally started from single spores and thus were considered to exhibit high genetic uniformity (but see Angelard et al. 2010). In the “combined” treatment, we halved inoculum amounts so that seedlings received equal amounts of both AM fungal species. Plants grew in autoclaved, nutrient-poor dune sand (Scheublin et al. 2007), with the following characteristics: pH 7.2; organic matter: 0.2%; P: 0.3 mg kg⁻¹ (CaCl₂-extracted); total N: 190 mg kg⁻¹. We maintained soil humidity at 70% water-holding capacity, and we added nutrients biweekly—8 mL of 1/2 P Hoagland solution (Arnon and Hoagland 1940) per pot. We randomized pots biweekly on benches, and they grew for 12 weeks per generation under semicontrolled light intensity with a 16L : 8D photoperiod.

We grew plants for three consecutive seasons. After the first generation, we randomly assigned the soils to either a mixed soil treatment (reduced spatial structuring, i.e., low population structure) or nonmixed soil treatment (control spatial structuring, i.e., high population structure). In both treatments, we removed the central root with a core (2 cm × 14 cm), and replaced it with an equal core of sterile soil (±50 g). For the mixed treatment, we removed all soil and roots from the pots and cut the roots into ~1-cm pieces. Contents were then mixed and transferred back into their respective pots separately. In the nonmixed treatment, we left the soil and roots outside the core intact. We treated all pots with an excess of demineralized water to standardize for soil compactness and then planted a new seedling. We replicated each fungal treatment × soil treatment 12 times for a total of 72 pots. In the beginning of the third generation, one plant in the high-quality/nonmixed treatment died, resulting in only 11 biological replicates.

Plant and Fungal Measurements

After each generation, we harvested the aboveground plant material, which was oven-dried at 70°C for 72 h and

weighed. After the first generation, we determined P content with the molybdate blue ascorbic acid method following acid digestion (Watanabe and Olsen 1965) and N and C contents by dry combustion on an elemental analyzer (NC2500, ThermoQuest Italia, Rodana). For each pot, we removed four evenly spaced soil cores (same as used for removing central root system) that were replaced by sterilized sand. Half the cores were freeze-dried overnight, and the other half were used to measure the mycorrhizal root colonization, using trypan blue staining and the magnified intersection method (McGonigle et al. 1990; 100 intersections per replicate).

To compare the relative abundance of competing fungal species in the combined fungal treatments, we performed DNA analyses after each generation and measured the fungal abundances in the single-species treatments after the third generation. We pooled roots from two cores per replicate, weighed and ground them in a microcentrifuge tube using a 4-mm glass bead in an FP120 bead beater (ThermoSavant, Holbrook, NY; 4.0 m s⁻¹, 2 × 10 s). We spiked each sample with 2 × 10¹⁰ copies of a plasmid containing the cassava mosaic virus sequence motif. This served as an internal standard in order to exclude effects of variations in isolation efficiency and DNA degradation (see appendix). We extracted DNA from the resulting mixture using the DNeasy Plant Mini Kit (Qiagen, Hilden) and following the standard protocol and eluted in 100 μL of the provided elution solution. We analyzed the abundance of each species by quantitative polymerase chain reaction primers targeting the mitochondrial large subunit (mtLSU) rRNA gene (Kiers et al. 2011). These primers were specifically designed to quantify the mtLSU copy numbers of each species and have been found to be highly specific and accurate (for design, optimization, and validation, see appendix).

Statistical Analyses

We performed all statistical analyses using SPSS, version 17.0. To establish the effects of the factor “species” (with levels high quality, low quality, and combined) on plant biomass, we used MANOVA to test N content, P content, and hyphal colonization of roots (hyphal colonization of roots was arcsine transformed to meet assumptions). Then, in order to assess which of these response variables contributes most to differences between inoculation treatments, we performed a discriminant analysis. To test whether response variables were significantly different between plants inoculated with different species, we performed a one-way ANOVA for each response variable separately, followed by Tukey’s *b* post hoc test and pairwise ANOVAs for each of three species treatments.

We performed separate ANOVAs for shoot biomass dif-

ferences in mixed and nonmixed pots between first and final generation for the combined treatment. We performed an ANOVA on colonization responses (i.e., percentage root colonized by hyphae, arbuscules, or vesicles) as an indication of differences in the abundance of fungal nutrient transfer structures (e.g., arbuscules) versus storage structures (e.g., vesicles; Johnson et al. 2010) in the final generation. For the single-species treatments, we also compared relative abundance of arbuscules compared to vesicles for both mixed and nonmixed treatments, which were ln transformed to meet assumptions for ANOVA. We analyzed qPCR-derived abundance data of each species for the high-quality, low-quality, and combined fungal treatment between mixed and nonmixed soil treatments using the nonparametric Mann-Whitney *U*-test because the requirement for a normal data distribution of ANOVA was not met. Ranked abundance of each strain did not significantly deviate in their homogeneity of variance, which is a requirement of the Mann-Whitney *U*-test (Ruxton and Beauchamp 2008). In the case of no detection, we replaced species abundance with the detection level after confirming that these cases did not appear to represent statistical outliers.

Results

Benefits Conferred by Fungal Species to Host Plants

Plant growth and nutritional status after the first generation confirmed that the two fungal species differed in mutualistic benefits conferred to their hosts. This is consistent with previous plant growth results that utilized these fungal species (Kiers et al. 2011). MANOVA indicated a significant treatment effect on plant responses (Pillai's trace: $V = .98$, $F_{8,134} = 4.64$, $P < .001$), and discriminant analysis suggested an especially high difference between the low-quality mutualist versus the high-quality mutualist and combined treatment (appendix). Aboveground biomass contributed most strongly to this separation, followed by shoot P content, hyphal colonization, and shoot N content (appendix).

Inoculation by the high-quality mutualist (*Glomus intraradices*) resulted in significantly higher aboveground biomass ($F_{1,46} = 18.15$, $P = .001$) and shoot P content ($F_{1,46} = 10.92$, $P = .002$) than the low-quality mutualist (*Glomus custos*; fig. 2). The plants that were inoculated with both species (combined treatment) did not differ from plants that were only inoculated with the high-quality species *G. intraradices* (biomass: $F_{1,46} = 0.40$, $P = .531$; P content: $F_{1,46} = 0.20$, $P = .661$; fig. 2). We found no significant differences in plant N content ($F_{2,69} = 0.34$, $P = .710$), suggesting that biomass differences are predominantly the result of an improved P nutritional status and/or lower C

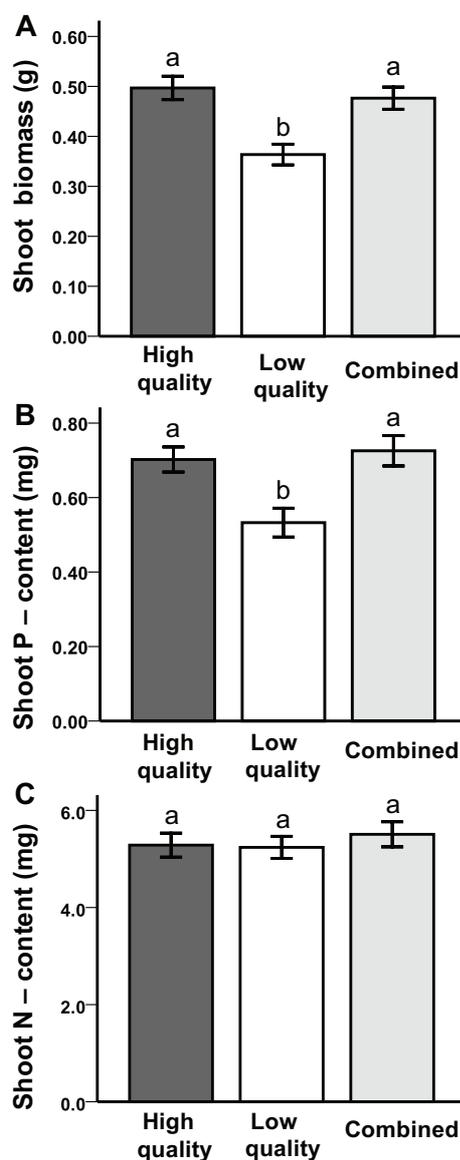


Figure 2: Effect of fungal treatments on shoot biomass and P content. Shoot biomass (A) and P content (B) of plants from generation one were significantly higher for plants inoculated with the high-quality species than with the low-quality species. For N content (C) there were no significant differences between treatments. When inoculated with both arbuscular mycorrhizal (AM) fungal species ("combined treatment"), the host plant biomass did not differ significantly from plants that were only inoculated with the high-quality species. Bars represent mean \pm 1 SE. Different letters represent significant differences according to Tukey's *b* post hoc test.

costs. The hyphal colonization levels of the high-quality and low-quality species in the single-inoculation treatments were high ($80\% \pm 1.8\%$ SEM vs. $71\% \pm 3.3\%$ SEM, respectively; henceforth all error estimates represent 1 SEM unless

stated otherwise), confirming that the host was able to form effective associations with both partners. Although these colonization levels differed significantly ($F_{1,46} = 6.67$, $P = .013$), the high colonization (above 70%) in each treatment and its low contribution to treatment separation in discriminant analysis (appendix) suggest that differences in mycorrhizal colonization were not the primary cause for differences in host benefit.

Mixed versus Nonmixed Soil, Effects on AMF Mutualists

We found that soil mixing significantly affected the success of competing high- and low-quality mutualists. In the combined treatment, the abundance of the low-quality mutualists was significantly reduced after two consecutive generations of soil mixing ($Z_{1,22} = -2.83$, $P = .004$, fig. 3). This effect was not due to a higher tolerance to soil mixing by the high-quality species. In the single-species treatments, mixing reduced the abundance of the high-quality species (from $8.2[\pm 1.4] \times 10^7$ to $4.4[\pm 0.8] \times 10^7$ marker copies g^{-1} root; $Z_{1,21} = -2.28$, $P = .023$), whereas

there was no significant negative effect of mixing on the abundance of the low-quality species in the single-species treatment. There was even a marginal positive effect of soil mixing on the low-quality species when grown alone (from $6.2[\pm 0.4] \times 10^6$ to $10.5[\pm 2.3] \times 10^6$ marker copies g^{-1} root; $Z_{1,22} = -1.67$, $P = .083$).

Plant Benefit under Mixed and Nonmixed Conditions

When both high- and low-quality fungal mutualists were present, soil mixing had a marginally significant positive effect on host shoot biomass compared to nonmixed conditions ($F_{1,22} = 3.40$, $P = .079$; fig. 4A). A positive effect of soil mixing on the symbiosis is further supported by the finding that in host roots, under mixed conditions, more arbuscules and fewer vesicles were formed than under nonmixed conditions, whereas there was no difference in hyphal colonization (arbuscules: $F_{1,22} = 8.77$, $P = .007$; vesicles: $F_{1,22} = 7.92$, $P = .010$; hyphae: $F_{1,22} = 0.03$, $P = .860$; fig. 4B). A high relative abundance of arbuscules (structures important for nutrient transfer) com-

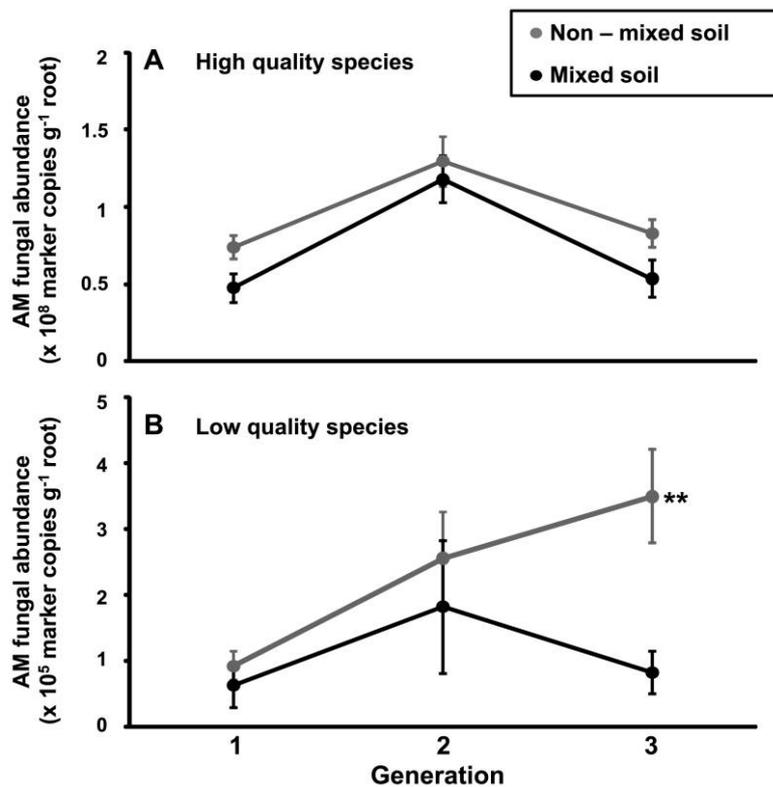


Figure 3: Effect of soil treatments on abundance of the fungal species, high-quality *Glomus intraradices* (A) and low-quality *Glomus custos* (B) in the “combined” treatment for each. In generation 3, the abundance of the low-quality species after mixing was significantly reduced compared to the nonmixed control. Error bars represent 1 standard error. Significant differences between mixed versus nonmixed are indicated with asterisks (two asterisks: $P \leq .01$). Note that mixing occurred twice (generation 1 to 2 and generation 2 to 3).

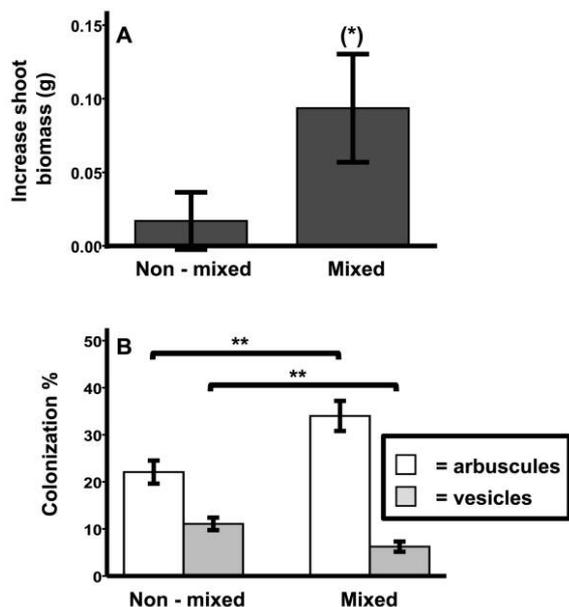


Figure 4: Effect of soil mixing on plant biomass and mycorrhizal colonization in “combined” treatment. Host plant increase in biomass from first to third generation when both AMF species were present (A) was marginally significantly larger (i.e., one asterisk: $P = .079$) when soil was mixed. This effect was associated (B) with a significant increase of arbuscular and decrease of vesicular colonization. Asterisks indicate significant differences (two asterisks: $P \leq .01$). Hyphal colonization did not differ between treatments (not shown). Bars indicate mean \pm 1 SE.

pared to vesicles (structures for storage) has been shown to be a useful indicator for mutualistic quality of AMF (Johnson et al. 2003, 2010). This higher arbuscule abundance could indicate increased colonization by the high-quality species because we found that high arbuscule abundance (compared to vesicle abundance) is a consistent identifiable trait of the high-quality species under both mixed ($F_{1,22} = 42.28$, $P = .001$) and nonmixed conditions ($F_{1,21} = 40.38$, $P = .001$).

Discussion

Spatial structure is frequently assumed to favor cooperation within mutualistic interactions (Frank 1994; Doebeli and Knowlton 1998; Bever and Simms 2000; Hoeksema and Kummel 2003; Foster and Wenseleers 2006; Gardner et al. 2007; Lion and van Baalen 2008; Platt and Bever 2009; Hodge et al. 2010). Other models (e.g., Sherratt and Roberts 2002) have found that cooperation can be maintained even in the absence of spatial structure depending on investment-response rules. Our model predicts that spatial structuring can indeed have contrasting effects on

selection for cooperation, depending on the scale (fine or coarse) at which hosts enforce cooperation (fig. 1). We tested these predictions using the arbuscular mycorrhizal symbiosis as a model system. We found that when we mixed the soil (reduced spatial structuring) in the combined treatment, this decreased the success of the low-quality species (fig. 3B). In contrast, mixing soil had no significant effect on the success of the high-quality species (fig. 3A).

There is a large theoretical literature examining how spatial structure would influence the evolution of cooperation. These include models for cooperation both between (see above) and within (reviewed by Lehmann and Rousset 2010; Nowak et al. 2010) species. Within species, population structuring keeps relatives together and hence can favor cooperation through kin selection (Hamilton 1964), as long as this is not negated by increased competition between relatives (West et al. 2002a; Lehmann and Rousset 2010). Between species, population structure can generate spatial correlations in the tendency to cooperate, which can favor cooperation (Frank 1994). In all cases, a general feature is that population structuring usually favors cooperation because of its tendency to create genetic correlations either within or between species (for an exception, see Hauert and Doebeli 2004). We gain the opposite prediction, because in our model, population structure influences the evolution of cooperation in a very different way. Specifically, it alters the extent to which one partner is able to preferentially reward more cooperative partners.

We used soil mixing as a mechanism to decrease the extent to which fungi populations are structured (i.e., to produce a lower F_{st}). Our model predicts that if plants can enforce cooperation at a fine scale, then lower spatial structuring will be relatively detrimental to the success of low-quality species (fig. 1). This effect arises because it will lead to patches containing both high- and low-quality mutualists, which facilitates the extent to which plants can differentially allocate resources to high-quality mutualists. Our empirical results are consistent with this model prediction (fig. 3B), although there may be other explanations (see discussion below). We also found that soil mixing led to a small increase in benefit for the host itself (fig. 4A). This benefit likely arose because the host was associating predominately with the high-quality species.

Our work suggests that if hosts are able to enforce cooperation on a fine scale, then high spatial structuring of the symbiont community may not be critical for stabilizing the partnership, at least in the model-system tested here. Recent work has highlighted potential mechanisms plants may employ to enforce cooperation at the fine scale assumed in our model (e.g., level of arbuscule). For example, stunted arbuscule morphology has been described when arbuscular-specific plant phosphate transporters were

knocked out (Javot et al. 2007), suggesting that when fungal symbionts fail to deliver P, plant hosts may reduce C allocation locally (Parniske 2008) or even digest individual arbuscles (Kobae and Hata 2010). Other work has pointed to the role of lysophosphatidylcholine, a compound that may help hosts to sense P concentrations, potentially allowing hosts to evaluate the amount of P delivered via the mycorrhizal pathway (Bucher et al. 2009). Now more work is needed to establish how plants physically control C transport across individual interfaces.

Are there alternative explanations for how soil mixing could favor the spread of the high-quality symbiont? Other differences between the species, such as ability to tolerate disturbance, could explain why the high-quality symbiont responds better to mixing. However, we can tentatively reject this hypothesis because the low-quality species appears to have greater inherent tolerance of mixing. In the single-species treatments, mixing reduced the abundance of the high-quality species (decrease of ~50%; $P = .023$), whereas mixing had no significant effect (even a small mean increase of ~60%; $P = .083$) on the low-quality species.

A second way in which soil mixing could favor one symbiont over the other would be through changes in the competitive dynamics between the two species not related to host C rewards. For instance, variation in life-history traits could mean that one species proliferates more, or earlier than the other, depending on our experimental conditions. However, by choosing closely related species, we reduced the variation associated with diverse life-history strategies typically found across genera (Denison and Kiers 2011). Future work should aim to extend these types of multigenerational tests to more fungal species pairs so broader conclusions can be drawn.

One important assumption of our work was that the control treatment, without soil mixing, showed a higher level of spatial structuring than the mixed treatment. Spatial structuring of AM fungal communities can arise from differential soil colonization patterns of strains (Smith et al. 2000; Oehl et al. 2005) or clustered sporulation (Jansa et al. 2002). In line with previous recommendations (e.g., Bever et al. 2009), we examined fungal competition under relatively realistic spatial conditions, that is, the structuring that originates during one or two generations of a successful symbiotic interaction (nonmixed) compared to a reduction of that structuring (mixed). We assume that even our nonmixed soil treatment was exposed to some mixing from root forging and other processes. No soil in nature would ever be free from mixing, especially at the fine level of the arbuscule. Manipulative tests of spatial structure should be extended to the field where natural processes such as nonrandom host-AMF associations (Öpik et al. 2009), burrowing animals (Mangan and Adler 2002), and fungal aggregation in response to plant sig-

naling cues (Yoneyama et al. 2008) can both increase and decrease spatial structure of symbiont communities.

Is there evidence for strong spatial structuring of symbiont communities in nature? While studies have demonstrated spatial structuring at the field or plot level (Mummey and Rillig 2008), the few studies that have examined spatial distribution of AM fungal species within individual plant roots, find fungal strains to be relatively well mixed (Alkan et al. 2006). This could be because relatively large root fragments (± 5 cm) were examined (Jansa et al. 2003) or small fragments were pooled (Wolfe et al. 2007). The smallest root fragments studied thus far (~1 cm), to our knowledge, did not reveal strong structuring (van Tuinen et al. 1998; Janouskova et al. 2009). A quantitative approach (e.g., Alkan et al. 2006) in which strain abundance (rather than presence-absence) is measured in small root fragments, will be the most successful at determining the degree of spatial structuring of AM fungal communities.

We found that the low-quality mutualist was not completely replaced by the high-quality mutualist. If the experiment had been continued for more generations, the abundance of the low-quality mutualist would have likely decreased further. However, our results suggest that the high-quality mutualist does not completely replace the other. One explanation is that host plants are physically unable to exclude colonization of a particular AM fungal species. Another explanation is that some fungi may provide benefits not measured (e.g., defense against pathogens; Herre et al. 2007), or provide benefits only under different conditions, and thus even putative low-quality mutualists are retained in low abundances (Smith et al. 2009; Palmer et al. 2010).

Can our model be extended to other systems? It would be interesting to test our model predictions in the legume-rhizobia mutualism, where fine-scale rewarding (at the level of the individual nodule) is known to operate (Kiers et al. 2006; Simms et al. 2006; Oono et al. 2011). Like in the fungal system, we would predict that soil mixing has the potential to be positive (or at least not negative) on the success of the high-quality strain by exposing the host to a greater diversity of potential partners. The only complication is that "mixed nodules" (containing more than one rhizobial strain) are known to occur with some frequency in the field (e.g., 12%–32%; Moawad and Schmidt 1987). There is currently no evidence that hosts can differentially control resource distribution to strains within a single nodule (Denison and Kiers 2011). So, if soil mixing also increased the frequency of mixed nodules, this could undercut any positive benefit for the host plant (Kiers et al. 2002; Friesen and Mathias 2010). Till and no-till agricultural systems could be useful for field-level experimental tests of our predictions, especially in cases where

high-quality fungal and rhizobial inoculum have been introduced. We predict that tillage could have a positive effect in promoting cooperation in the mycorrhizal symbiosis, but this effect is likely overridden by life-history differences among fungi in respect to their ability to handle extreme disturbances (Verbruggen and Kiers 2010).

More generally, whether spatial structure will stimulate or decrease mutualistic cooperation depends on the biology of the interaction. If a mutualism depends upon partners cooperating to produce a beneficial common good, without a mechanism to reward more cooperative partners (or punish less cooperative partners), then increased spatial structure favors cooperation, because it increases relatedness among symbionts leading to a shared interest between symbiont and host (Frank 1994; Bever and Simms 2000; West et al. 2002b; Foster and Wenseleers 2006). This is analogous to how spatial structuring favors cooperation within species (Griffin et al. 2004; Diggle et al. 2007; Cornwallis et al. 2009; Lehmann and Rousset 2010). In other symbiotic interactions, like the mycorrhizal symbiosis, mixing may increase the potential suite of partners available to host plants. This could increase host plant benefit as long as plants can discriminate among partners at a fine scale. Research is now needed to (i) identify potential physiological mechanisms that would allow plants to control allocation processes and (ii) test these dynamics in the field to determine the actual effect of symbiont mixing under natural conditions.

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APPENDIX

qPCR Methodology and Multivariate Statistics

Design of qPCR Markers

Primers with hydrolysis probes designed for specific detection and quantification of *Glomus intraradices* and *Glomus custos* (isolates obtained from Mycovitro Biotechnología ecológica), targeting the mitochondrial large subunit

RNA gene, were designed taking the following steps: DNA was extracted from AM fungi spores produced monoxenically with carrot root organ cultures or from the colonized carrot roots from the same cultures, using the DNeasy Plant Mini kit (Qiagen, Hombrechtikon), following manufacturer recommendations. The final volume of the DNA preparations from spores was 20 μ L (instead of 100 μ L recommended by the manufacturer) to maximize DNA concentrations before PCR. The roots samples were eluted with 100 μ L of the elution buffer. Monoxenic fungal cultures were provided by A. Bago, H. Bücking, and M. Hart.

DNA was subjected to polymerase chain reaction (PCR) amplification of the mitochondrial large ribosomal subunit (mtLSU) RNA gene with following primer pair combinations, according to Börstler et al (2008): RNL11-RNL17, RNL1-RNL14, or RNL1-RNL15. The PCR was carried out using Taq PCR Core kit with CoralLoad reaction buffer (Qiagen), using 25- μ L PCR reaction volume, 1- μ M concentration of each primer, and 38 cycles (denaturation at 95°C for 10 s, annealing at 50°C for 90 s, and amplification at 72°C for 90 s). Amplified DNA fragments were cloned into a blue-script vector (pGEM-T Easy vector system; Promega, Dübendorf) and sequenced at Microsynth (Balgach). The sequences were individually edited and the clones resequenced if quality of the reads was insufficient. The identity of the sequences was revealed by BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to sort out contaminant sequences (e.g., bacteria, unspecific amplifications of other genome regions). Several partial sequences of the mitochondrial large subunit of the different AMF species were obtained, which were deposited in the GenBank under the accession numbers HQ706096–HQ706103.

These sequences were aligned with other mtLSU sequences available in the GenBank (e.g., *G. intraradices*, *Glomus proliferum*, *Glomus clarum*) and at least two combinations per AMF species of species-discriminating qPCR primers with associated hydrolysis probes were designed using AlleleID software (ver. 6; Premier Biosoft International, Palo Alto, CA). Care was taken to target mtLSU regions coding for the ribosomal RNA, that is, to avoid putative introns described recently by Thiery et al. (2010). Specificity of the primers and fluorescent probes was confirmed with a BLAST search and the oligonucleotides (primers and dually labeled hydrolysis probes, labeled with fluorescein at the 5' end and BHQ-1 quencher at the 3' end) were then synthesized in Microsynth. The primers were purified by preparative HPLC and the probes by preparative polyacrylamide gel electrophoresis before lyophilization. Both primers and probes were diluted with PCR-grade water to achieve 25- μ M concentrations, aliquoted (20 μ L each), and frozen at -20° C.

Table A1: qPCR markers for specific quantification of *Glomus intraradices* and *Glomus custos* by means of measuring gene copies of the mitochondrial large ribosomal subunit of the respective arbuscular mycorrhizal fungi species (see Kiers et al. 2011)

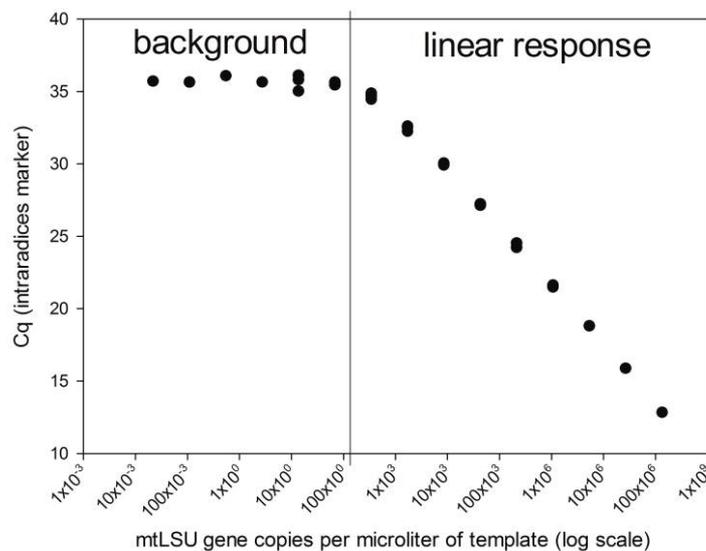
| Marker | Target | Sequences 5'→3' (forward primer, reverse primer, hydrolysis probe) | No. cycles | Denaturation (°C/s) | Annealing (°C/s) | Amplification (°C/s) |
|-----------|------------------------|--|------------|---------------------|------------------|----------------------|
| Intra mt5 | <i>G. intraradices</i> | TTTTAGCGATAGCGTAACAGC, TACATCTAGGACAGGGTTTCG, FAM-AAACTGCCACTCCCTCCATA-TCCAA-BHQ1 | 65 | 95/10 | 60/10 | 72/1 |
| cust | <i>G. custos</i> | TCTAACCCAGAAATGTATAG, AAGGACTGCCTTGTGTTC, FAM-ATACAATAATGGGCAATCAGACATATCGT-BHQ1 | 65 | 95/10 | 62/15 | 72 /1 |
| CMV | Cassava mosaic virus | CGAACCTGGACTGTTATGATG, AATAACAATCCCCTGTATTTTCAC, FAM-CACCAGGCACCAACAACGAC-CATT-BHQ1 | 45 | 95/10 | 50/30 | 72/1 |

Note: Also represented are the markers for partial cassava mosaic virus DNA sequence (Genbank accession number AJ427910; inserted in vector pUC19) quantification, used as an internal DNA standard in the experiment. **FAM** = fluorescein; **BHQ1** = fluorescence quencher.

Marker Selection, Optimization of Cycling Conditions, Cross-Reactivity Testing

First, the markers were tested for their specificity under low stringency cycling conditions (denaturation at 95°C for 10 s, annealing at 52°C for 30 s, and amplification at 72°C for 5 s). In this assay, DNA extracts from *Medicago truncatula* roots colonized by the different AMF (three individual samples per each AMF species) were used as templates. Second, markers showing greatest specificity to-

wards their target species (either no cross-amplification with other species or the greatest difference in detection cycle (C_q) value between target and nontarget species) were selected for further optimization (table A1). Stringency of cycling conditions was then increased stepwise for each of the markers to achieve a complete avoidance of amplification with nontarget samples (see table A1 for details of the optimized cycling conditions). The markers were confirmed to amplify their target AMF species and to avoid nontarget AMF species as well as the plant DNA

**Figure A1:** Typical response of qPCR assay to DNA template dilution. Linear response region contains values used for calibration of the qPCR assay and the background region has been used for assessment of detection limit of the qPCR assay (see Kiers et al. 2011).

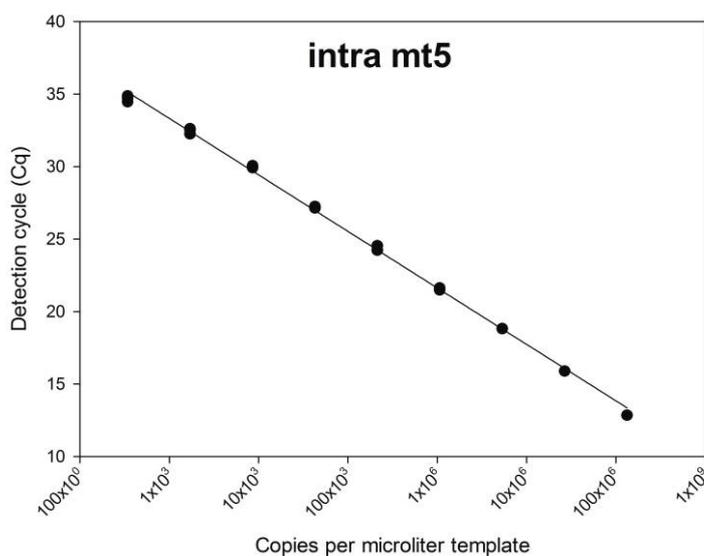


Figure A2: Calibration curves for the qPCR assays targeting the mitochondrial large subunit (mtLSU) of *Glomus intraradices* and *Glomus custos*. Equations for conversion of the qPCR signal to mtLSU copy concentrations in template are given for each assay. Term CP stands for number of target gene copies per microliter template (*G. intraradices*: $R^2 = 0.998$, $CP = 10 \times (47.33655877 - Cq)/4.22866042$; *G. custos*: $R^2 = 0.999$, $CP = 10 \times (39.38265200 - Cq)/3.74460986$; see Kiers et al. 2011).

(tested on spores and colonized roots of *M. truncatula* and *Daucus carota*). All qPCR assays were carried out in 9- μ L reaction format, using the LightCycler 2.0, LightCycler TaqMan chemistry (LightCycler TaqMan Master, cat. 04735536001), and 20- μ L Lightcycler glass capillaries (cat. 04929292001). Final concentration of the primers was 0.5 μ M each, and the final concentration of the hydrolysis probe was 0.11 μ M. A 2.25- μ L DNA template (i.e., sample) was included in each reaction.

qPCR Calibration, Detection Limits

Two to four individual plasmid preparations per AMF species were used for calibration of the qPCR markers. These plasmids were carrying fragments of mtLSU of the respective AMF species with 100% sequence match to the region amplified by the markers (confirmed by sequencing). Plasmids were isolated from overnight cultures of the transformed *Escherichia coli* JM109 (Promega), grown on LB medium supplemented with ampicillin (100 μ g mL^{-1}), using the Miniprep procedure (Sambrook et al. 1989). The plasmids were linearized using EcoRI⁺ (Fermentas, Le Mont-sur-Lausanne) digestion, incubating the samples at 37°C for 2 h and then at 65°C for 20 min. The concentration of the DNA was then measured by PicoGreen fluorescence assay (Invitrogen P7589), using LightCycler 2.0 at 45°C and measuring the emission at 530 nm. The concentration of plasmid copies per unit of sample volume

was calculated according to Jansa et al. (2008), by knowing the concentration of DNA in each sample, length of insert (176 bp for *G. intraradices* and 438 bp for *G. custos*), and length of vector (3,015 bp), and by assuming molecular weight per nucleotide double-stranded DNA to be 660 Da. Plasmid preparations were serially diluted (fivefold and 10-fold) to achieve a range of plasmid concentrations from a few billions per microliter to less than 1 μL^{-1} . These were used for establishment of (1) calibration curves for conversion of the qPCR detection cycle (Cq) to gene copy concentrations and (2) assessment of detection limits of the qPCR markers. That is, the Cq is typically negatively and linearly correlated to the log-transformed template concentrations (fig. A1, linear response region), until the detection limit of the assay is reached and the Cq becomes independent of the further dilution (fig. A1, background region) or there is no response at all.

Linear response region of each of the calibration assay has been used to derive equations allowing conversion of

Table A2: Detection limits and minimal detectable target gene concentrations of the two qPCR assays

| Marker system | Detection limit (Cq) | Threshold mtLSU gene copy concentration (copies μL^{-1}) |
|---------------|--------------------------|--|
| Intra mt5 | 37.62 | 199 |
| Cust | 35.6 | 10 |

Cq values to mtLSU gene copies per unit volume of the template (fig. A2). Detection limits were derived from the background region of the qPCR response curve $DL = AV_{Cq(\text{back})} - 3 \times SD(AV_{Cq(\text{back})})$, where DL is the detection limit of the assay (Cq value), $AV_{Cq(\text{back})}$ is the mean of the Cq values in the background region, and $SD(AV_{Cq(\text{back})})$ is the standard deviation of this mean. The detection limits of the two marker systems and corresponding threshold mtLSU concentrations are given in table A2. These assays were then used for assessment of mtLSU gene copy concentrations in DNA samples, taking into account any dilutions of the template during the sample processing.

MANOVA and Discriminant Analysis

Multivariate analysis of variance (MANOVA) was performed to assess the effect of our treatments (high quality, low quality, and both strains combined) on the following response variables: aboveground biomass, shoot P content, shoot N content, and (arcsine-transformed) hyphal colonization of roots. The other colonization variables (arbuscules and vesicles) were not included, because even after transformation they still deviated from normal distribution (for the first generation of the experiment). Using Pillai's trace, we found a significant effect of our treatments: $V = .98$, $F_{8,134} = 4.64$, $P < .001$.

We used discriminant analysis to assess which of the response variables (plant biomass, P, N, and hyphal colonization) best distinguished our treatments after the first generation. The first discriminant function was found to explain 94.8% of variance, with a canonical R^2 of 0.40. The second explained only 5.2% of variance, canonical $R^2 = .035$, and therefore the first function had by far the strongest predictive power. In line with this, only the first discriminant function significantly differentiated the treatments (thus high-quality, low-quality, and both strains combined) $\Lambda = .58$, $\chi^2 = 36.7$, $P < .001$.

Of the response variables assessed, aboveground biomass had the highest loading on the first function ($r = .68$) followed by shoot P content ($r = .55$), hyphal colonization of roots ($r = .42$), and shoot N content ($r = .06$). The mean scores of treatments (group centroids) for the first function were low quality: -1.1 , combined: 0.36 , and high quality: 0.75 , indicating especially a strong separation of low-quality versus combined and high-quality treatments, with low quality and high quality at the extremes.

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