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31 **response to increasing soil fertility**
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53 **Abstract**
54
55 Understanding the underlying mechanisms driving responses of belowground
56 communities to increasing soil fertility will facilitate predictions of ecosystem
57 responses to anthropogenic eutrophication of terrestrial systems. We studied the
58 impact of fertilization of an alpine meadow on arbuscular mycorrhizal (AM) fungi, a
59 group of root-associated microorganisms that are important in maintaining sustainable
60 ecosystems. Species and phylogenetic composition of AM fungal communities in
61 soils were compared across a soil fertility gradient generated by 8 years of combined
62 nitrogen and phosphorus fertilization. Phylogenetic patterns were used to infer the
63 ecological processes structuring the fungal communities. We identified 37 AM fungal
64 virtual taxa, mostly in the genus *Glomus*. High fertilizer treatments caused a dramatic
65 loss of *Glomus* species, but a significant increase in genus richness and a shift towards
66 dominance of the lineage of *Diversispora*. AM fungal communities were
67 phylogenetically clustered in unfertilized soil, random in the low fertilizer treatment
68 and over-dispersed in the high fertilizer treatments, suggesting that the primary
69 ecological process structuring communities shifted from environmental filtering
70 (selection by host plants and fungal niches) to a stochastic process and finally to
71 competitive exclusion across the fertilization gradient. Our findings elucidate the
72 community shifts associated with increased soil fertility, and suggest that high
73 fertilizer inputs may change the dominant ecological processes responsible for the
74 assembly of AM fungal communities towards increased competition as photosynthate
75 from host plants becomes an increasingly limited resource.
76
77 **Keywords:** Symbiotic fungi; Phylogenetic structure; Ecological process; Competition;
78 Environmental filtering; Resource availability

## 1. Introduction

Worldwide increases in fertilizer application and nutrient deposition raise the question of how nutrient enriched soil influences biotic communities. Numerous field studies have shown that improved soil fertility increases plant productivity, but also decreases plant species diversity and changes plant community composition mainly through the enhanced process of competitive exclusion (Rajaniemi, 2002; Hautier et al., 2009; Borer et al., 2014; Liu et al., 2015). Diverse belowground communities of microorganisms interact with plants and serve as key players in biogeochemical cycling (Fitter et al., 2005; Philippot et al., 2013), but in comparison with that of plant community, relative little is known about the patterns and underlying mechanisms of belowground community responses to soil nutrient enrichments. Filling this knowledge gap can help guide management decisions and facilitate predictions of ecosystem sustainability in a changing world.

Arbuscular mycorrhizal (AM) fungi in the phylum Glomeromycota are widespread root-associated microorganisms that are known to be sensitive to fertilization. In general, plants supply associated AM fungi with carbohydrates, and in return, fungi provide soil phosphorus (P) and possibly nitrogen (N) to their host plants (Hodge et al., 2010; Selosse and Rousset, 2011). Mycorrhizal symbioses can also provide plants with other benefits such as protection against root pathogens (Lewandowski et al., 2013) and several types of abiotic stress (Aroca et al., 2007). Furthermore, increasing evidence shows that mycorrhizas influence the structure of plant communities (Klironomos et al., 2011; Yang et al., 2014), the rhizosphere microbiome (Vestergård et al., 2008; Veresoglou et al., 2012), soil structure (van der Heijden et al., 2006; Leifheit et al., 2015), and nutrient cycles (Cheng et al., 2012; Bender et al., 2015). Many experiments have shown that fertilization reduces AM fungal diversity and shifts the community toward dominance of particular species or lineages (e.g. Johnson, 1993; Egerton-Warburton et al., 2007; Alguacil et al., 2010; Liu et al., 2012, 2015; Camenzind et al., 2014). However, neutral or even positive effects of fertilization on AM fungal diversity have also been reported (e.g. Antoninka et al., 2011; van Diepen et al., 2011; Gosling et al., 2013), suggesting that the responses of AM fungal communities to fertilization are influenced by complex interactions of many factors (van Diepen et al., 2011; Vályi et al., 2015).

Although it has long been recognized that fertilization influences the structure of

113 AM fungal communities, the mechanisms responsible for these impacts remain
114 largely unclear. In theory, there are several possible mechanisms by which increasing
115 soil fertility may influence the composition of AM fungal communities including
116 competition among AM fungi and environmental filtering because of fungal
117 preferences or host plant selection. First, fertilization may enhance competition
118 among coexisting AM fungi because plants generally reduce the amount of
119 carbohydrate supplied to mycorrhiza when they are not limited by soil nutrients
120 (Johnson, 2010; Olsson et al., 2010); and, enhanced competition may result in loss of
121 fungal diversity and a shift of the species composition towards dominance of AM
122 fungi that are superior competitors when carbohydrates are limited (Hepper et al.,
123 1988; Johnson, 1993). Second, because species of AM fungi have different niches and
124 are known to prefer to inhabit different soil conditions (Schechter and Bruns, 2008;
125 Alguacil et al., 2010; Dumbrell et al., 2010), fertilization may directly select taxa that
126 grow best in the enriched conditions. Third, there is good evidence that plants actively
127 select AM fungal taxa that best provision nutrients (Parniske, 2008; Kiers et al., 2011;
128 Bever, 2015) and many field studies have shown that certain plants select particular
129 AM fungi (e.g. Vandenkoornhuyse et al., 2003; Veresoglou and Rillig, 2014); thus,
130 the loss of plant species caused by fertilization can lead to significant changes in the
131 AM fungal community (Liu et al., 2012).

132 Analysis of phylogenetic structure has been used to infer the relative importance
133 of competitive exclusion, environmental filtering, or stochastic process in driving
134 community assembly (Webb et al., 2002). If the phylogenetic niches of organisms are
135 conserved such that more closely related taxa have similar niche-related traits, then
136 competitive exclusion and environmental filtering will generate patterns of over-
137 dispersion and phylogenetic clustering, respectively (Webb et al., 2002). This method
138 was first used in the studies of plant communities (e.g. Webb, 2000; Dinnage, 2009;
139 de Freitas et al., 2014; Parmentier et al., 2014), but is now increasingly used to
140 analyze the community assembly of mycorrhizal fungi (e.g. Lim and Berbee, 2013;
141 Grilli et al., 2014; Horn et al., 2014; Rincón et al., 2014; Shi et al., 2014). A factorial
142 field study of the influence of light and nutrient availability analyzed the phylogenetic
143 structure of AM fungal communities inside roots and determined that environmental
144 filtering structured communities under unfertilized conditions but random process was
145 most important under a low level of fertilization (Liu et al., 2015). The purpose of our
146 current study is to analyze the phylogenetic structure of AM fungal communities

147 across a long-term fertilization gradient and test the hypothesis that increasing
148 fertilization will cause plants to allocate less photosynthate to their fungal symbionts
149 and increase the relative importance of competitive interactions for structuring AM
150 fungal communities.

151 We have previously shown that eight years of combined N and P fertilization of
152 an alpine meadow reduces the abundance and diversity of AM fungal communities
153 inside roots (Liu et al., 2012). However, the ecological processes driving these
154 community shifts were not examined. Our current study uses community phylogenetic
155 analysis to study the AM fungal communities in the soil of the same fertilization
156 experimental system as Liu et al. (2012) to better understand the mechanisms by
157 which increasing soil fertility structures AM fungal communities. We chose to
158 identify communities from soil DNA rather than roots because soil contains both
159 active (extraradical hyphae) and dormant (spore) structures of AM fungi and thus may
160 reflect a larger proportion of the total species pool (Alguacil et al., 2014). We
161 predicted that, as in the root-derived results reported in Liu et al. (2012), increasing
162 soil fertility would significantly reduce the diversity of soil-borne AM fungi; and
163 additionally, this reduction should be largely attributed to the enhanced competition
164 process. In particular, the current study aimed to answer the following questions: (1)
165 how do species composition and phylogenetic structure of AM fungal communities
166 respond to a fertilization gradient? (2) Which is the primary ecological process
167 structuring AM fungal communities across a fertilization gradient?
168

169 **2. Materials and methods**

170

171 2.1. Description of study site and fertilization treatment

172

173 This study was conducted at the Walaka experimental site (34°00′N, 102°00′E;
174 3500 m above sea level) of the Research Station of Alpine Meadow and Wetland
175 Ecosystems of Lanzhou University in the eastern Qinghai-Tibetan Plateau of China.
176 This region is a typical alpine meadow ecosystem, where the mean annual
177 temperature is 1.2 °C, the mean annual precipitation is 620 mm, the dominant plants
178 consist mainly of Cyperaceae and Poaceae, the growing season is from May to
179 September, and the diversity of both plants and AM fungi is relatively high (Liu et al.,
180 2012). Since 2001, the experimental site has been fenced and only grazed by yak and

181 sheep every winter from October to April.

182 The long-term fertilization experiment, with 25 plots (5 rows by 5 columns) 183 consisted of five fertilization levels and five replicates using a randomized block 184 design. Plots were established on a flat area on March 2002. Each plot was $10 \times 6\text{-m}^2$ 185 and separated from the others by a 1-m buffer strip. Five fertilization treatments were 186 generated with 0, 30, 60, 90 and 120 g $m^{-2}$ $yr^{-1}$ of $(NH_4)_2HPO_4$ fertilizer applied 187 annually from 2002 at the beginning of the growing season, and these treatments are 188 hereafter referred to as F0, F30, F60, F90 and F120, respectively. The corresponding 189 N and P inputs of each treatment are as follows: F0, control; F30, 6.4 g N and 7 g P $m^{-2}$ 190 $yr^{-1}$; F60, 12.7 g N and 14.1 g P $m^{-2}$ $yr^{-1}$; F90, 19.1 g N and 21.1 g P $m^{-2}$ $yr^{-1}$; F120, 191 25.4 g N and 28.2 g P $m^{-2}$ $yr^{-1}$. After eight years of fertilization treatment (in 2010), 192 soil available N and P concentrations gradually increased across the fertilization 193 gradient, while plant species richness decreased dramatically and plant community 194 shifted towards dominance by *Elymus nutans* (Table S1). More details of the changes 195 in soil and plant properties across the fertilization gradient are described in Liu et al. 196 (2012).

197

198 2.2. Soil sampling and DNA extraction

199

200 Soil samples were collected on 20 May, 10 July and 5 September 2010, 201 concurrently with the samples collected for Liu et al. (2012). At each sampling date, 202 nine replicate soil cores (3.8 cm diameter, 25 cm depth) were collected randomly from 203 each plot and samples were manually homogenized. From each composite sample, 204 about 50 g root-free soil was subsampled and stored in a pre-sterilized aluminum 205 container and used for DNA extraction (75 soil samples in total). The remaining soil 206 and roots were analyzed in Liu et al. (2012). All samples were transported to the 207 laboratory within 36 h using an ice box.

208 Soil DNA was extracted from a 0.25-g subsample of each of the 75 soil samples 209 using the PowerSoil™ DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, USA) 210 according to the manufacturer's protocol. Extracted DNA was examined on 0.8% 211 (w/v) agarose gel with ethidium bromide staining to confirm the extracted efficiency. 212 All extracted DNA was stored at -80°C until further use.

213

214 2.3. Molecular analysis of AM fungal community

215

216 Before analysis, the soil DNA collected from each plot was combined across the
217 three sampling dates, resulting in 25 pooled DNA samples. The AM fungal
218 community in soil was analyzed by a PCR-cloning-sequencing approach using the
219 procedure and conditions described by Liu et al. (2012). Briefly, partial 18S rRNA
220 gene sequences of AM fungi were amplified from soil DNA via a nested PCR, using
221 GeoA2-Geo11 (Schwarzott and Schüßler, 2001) and NS31-AML2 (Simon et al., 1992;
222 Lee et al., 2008) as the first and second primer combinations, respectively. The second
223 PCR products were purified and used to construct clone libraries (25 in total), and
224 then the DNA inserts were re-amplified with NS31-AML2 (48 putative positive
225 clones per clone library) and screened using restriction fragment length polymorphism
226 (RFLP) with restriction enzymes *Hin*fI and *Hin*1II. The RFLP patterns were only
227 compared within the five replicate samples within the same fertilization treatment.
228 One representative clone of each RFLP type in each treatment was sequenced (179
229 sequences in total), and the remaining clones were classified by RFLP typing. All
230 DNA sequences were edited using the ContigExpress software (InforMax Inc., MD,
231 USA) and compared with published nucleotide sequences using the online BLAST
232 search tool (http://blast.ncbi.nlm.nih.gov; accessed 10 March 2011). Non-AM fungi
233 and possible chimeric sequences were eliminated from the dataset, and the remaining
234 136 AM fungal sequences were analyzed further. In total, 835 AM fungal clones were
235 identified from 1200 clones (*c.* 70%). All AM fungal sequences obtained in this study
236 have been deposited in the GenBank database under accession numbers JN009482-
237 JN009617.

238 To facilitate the comparison between our AM fungal sequences with other studies,
239 we delimited our sequences into different molecular virtual taxa (VTs, similar with
240 molecular operational taxonomic unit or phylotype) using the online Maarj*AM*
241 database (http://maarjam.botany.ut.ee; accessed 15 November 2013). This database
242 has collected most published 18S rRNA gene sequences of AM fungi and
243 phylogenetically defined sequence groups as AM fungal VTs with sequence identity
244 ≥97% (corresponding to species-level taxa roughly; Öpik et al., 2010). The VT
245 taxonomy of AM fungi is increasingly used in identification of environmental
246 sequences (e.g. Öpik et al., 2014; Xiang et al., 2014; Liu et al., 2015). After BLAST
247 against the Maarj*AM* database, each obtained sequence was grouped into a
248 corresponding VT according to the sequence identity (≥97%), query coverage (≥97%)

249 and BLAST bit score. To further confirm the BLAST-based VT delimitation, we
250 aligned our AM fungal sequences and the representative sequences of VTs in
251 Maarj*AM* database using ClusterW and constructed a maximum likelihood (ML) tree
252 using MEGA 5.0 with Tamura 3-parameter model and 1000 bootstrap replications
253 (Tamura et al., 2011). Some sequences with ambiguous VT delimitation (i.e. one
254 sequence could be grouped into more than one VT after BLAST search) were
255 corrected according to the sequence identity, bootstrap value and the topology of the
256 phylogenetic tree (Liu et al., 2015).
257 To elucidate the phylogenetic relationships between our VTs and published AM
258 fungal sequences, we constructed a ML phylogenetic tree using DNA sequences
259 including the representative sequences of our VTs, the representative sequences of
260 major AM fungal genera and the most closely related sequences from GenBank
261 database. Each VT obtained in this study was grouped into a corresponding AM
262 fungal genus according to the phylogenetic tree, and if a VT could not be placed in a
263 known genus, we regarded this as a new genus-like clade. The VT/genus
264 compositions of AM fungi were calculated on the basis of the clone numbers of each
265 VT/genus in each clone library.
266
267 2.4. Statistical analysis
268
269 As our sampling was concurrent with the sampling work reported in Liu et al.
270 (2012), the data of soil characteristics and plant variables used in this study were
271 derived from that study. Before analysis, a principal component analysis ('prcomp'
272 function of R package 'stats', R Core Team., 2015) was carried out using the ln(x+1)
273 transformed data of soil characteristics (including soil available N, available P, N:P
274 ratio, total N, organic C, C:N ratio, moisture and pH) in order to generate fewer
275 compound variables (principal components, PC) that characterize the soil properties.
276 PCA results show that 74% of total variance could be summarized in the first two
277 components (Fig. S1): the PC1 (44%) describes a main gradient of soil available N
278 (loading value = 0.47) and available P (0.44); the PC2 (30%) reflects a main gradient
279 of soil moisture (0.51) and organic carbon (0.50). In the following analyses, we used
280 the scores of PC1 and PC2 to assess the relationships between AM fungal variables
281 and soil properties.
282 Based on the community matrices of VT/genus compositions of AM fungi, the

283 VT/genus richness as well as the relative abundance of each VT/genus in each sample
284 were calculated, and the sampling effort curves of VT richness were examined using
285 'rarecurve' function of the R package 'vegan' (Oksanen et al., 2015). To test if the
286 spatial autocorrelations were present in our measured variables, we calculated the
287 Moran's I autocorrelation coefficients using 'Moran.I' function in R library 'ape'
288 (Paradis et al., 2004) with a matrix of plot distance weights and our AM fungal
289 variables. We did not observe significant spatial autocorrelations being present in all
290 tested AM fungal variables; thus, the effects of spatial autocorrelation were not taken
291 into account in below analyses. The effects of fertilization on AM fungal variables
292 were analyzed using a linear mixed effects model, with fertilization as a fixed effect
293 and block as a random effect. These analyses were conducted using the 'lme' (model
294 fitting) and 'anova.lme' (ANOVA summary of the linear mixed effects model)
295 functions in the R package 'nlme' (Pinheiro et al., 2015). Based on the 'lme' model,
296 we also summarized the post-hoc pairwise comparisions (method = Tukey) to
297 illustrate the differences of each variable between fertilization treatments using 'glht'
298 function of R package 'multcomp' (Hothorn et al., 2008). Relationships between AM
299 fungal variables with two extracted PCA components of soil properties, plant species
300 richness and the relative abundance of *Elymus nutans* were tested using correlation
301 and regression analyses (SPSS Inc., IL, USA).

302 The dissimilarity of communities of AM fungal VT across samples was analyzed
303 by non-metric multidimensional scaling (NMDS) with Bray-Curtis dissimilarity
304 measurement using 'metaMDS' function, and the treatments (represented by F0 to
305 F120) were fitted as centroids onto the two dimensional ordination plot using
306 'ordiellipse' function. Relationships between AM fungal community composition with
307 two extracted PCA components of soil properties, plant species richness and the
308 relative abundance of *Elymus nutans* were tested using 'envfit' function. All of these
309 statistical analyses were done using the R package 'vegan' (Oksanen et al., 2015).

310 The community phylogenetic structure was analyzed using the R package
311 'picante' (Kembel et al., 2010). We used the mean pairwise phylogenetic distance
312 (MPD) to quantify the relatedness of co-occurring AM fungal VTs within a
313 community (Webb et al., 2002; Horn et al., 2014). Based on the MPD, we calculated
314 the inter-community MPD (betaMPD) using 'comdist' function, and again we
315 performed NMDS and 'envfit' using the data of betaMPD. The difference in
316 community phylogenetic composition between a given pair of samples can be clearly

317 depicted on the two dimensional NMDS plot (Liu et al., 2015). To evaluate the degree
318 of non-random phylogenetic structure of our communities, we calculated the nearest
319 relative index (NRI) using 'ses.mpd' function (NRI is equivalent to -1 times the
320 output of 'ses.mdp'; Kembel et al., 2010), in which the MPD for each VT was
321 weighted by its abundance, and the observed MPD was compared with the null
322 distribution of MPD generated by the 1000 randomizations of 'phylogeny.pool' null
323 model. The NRI is a standardized measure of the mean pairwise phylogenetic distance
324 of taxa in a sample (Webb et al., 2002), and through which we could infer the
325 importance of niche-based and neutral processes in driving community assembly
326 (Kembel, 2009). In general, a mean NRI across all samples that is significantly greater
327 than zero is correlated with phylogenetic clustering, equal to zero with random, and
328 less than zero with over-dispersion (Kembel, 2009). The significant difference
329 between NRI and null expectation of zero was tested using two-tailed *T* test at the 95%
330 confidence level (SPSS Inc., IL, USA).
331
332 **3. Results**
333
334 3.1. AM fungal taxa detected in this study
335
336 In total, 37 AM fungal VTs were identified in all soil samples, of which eleven
337 VTs were related to sequences of morphologically described species (Table 1). These
338 VTs belong to nine known genera and two new genus-like clades within five families
339 (Fig. 1): Glomeraceae (including 27 VTs in 6 genera), Diversisporaceae (5 VTs in 2
340 genera), Claroideoglomereae (3 VTs in 1 genus), Archaeosporaceae (1 VT in 1 genus)
341 and Ambisporaceae (1 VT in 1 genus). Inspection of the VT accumulation curves
342 showed that the curves from F60, F90 and F120 were closer to asymptotic than the
343 curves from F0 and F30 (Fig. S2), suggesting that a higher proportion of the AM
344 fungal diversity was captured in high fertilizer treatments. Overall, VT62 (related to
345 an unknown *Diversispora* sequence; 16% of all AM fungal clones) and *Glomus* (38%)
346 were the most frequently detected VT and genus, respectively.
347
348 3.2. Fertilization effects on AM fungi
349
350 The relative abundance of most VTs varied among treatments (Table 1), with

351 some VTs decreased (e.g. VT222 and VT371) while other increased (e.g. VT62 and
352 VT 64) across the fertilization gradient. Similarly, the abundance of most AM fungal
353 genera, especially the *Glomus* (decreased by fertilization; $F_4$ = 24.74, $P$ < 0.001) and
354 *Diversispora* (increased by fertilization; $F_4$ = 18.08, $P$ < 0.001), were very sensitive to
355 fertilization treatment. The abundance of *Glomus* was negatively correlated with that
356 of *Diversispora* ($r$ = -0.653, $P$ < 0.001) and, the former significantly decreased but the
357 latter increased with increasing soil N and P fertility and the dominance of *Elymus*
358 *nutans* (Fig. 2a,c); moreover, the *Glomus* and *Diversispora* abundance were correlated
359 positively and negatively with plant species richness, respectively (Fig. 2d).

360 The VT richness of both all lineages ($F_4$ = 5.71, $P$ = 0.005) and *Glomus* ($F_4$ =
361 19.24, $P$ < 0.001) declined but genus richness increased ($F_4$ = 3.05, $P$ = 0.048) with
362 increasing fertilizer inputs (Fig. 3a,b), and all were correlated significantly with soil
363 PC1 scores and the abundance of *Elymus nutans* (all negatively with VT richness and
364 positively with genus richness; Table 2). NMDS ordinations revealed that both the VT
365 and phylogenetic compositions of AM fungal communities varied among treatments
366 (Fig. 4), with the exception of similar phylogenetic compositions under treatments of
367 unfertilized control and F30 (Fig. 4b). Both community ordinations were highly
368 related with plant species richness, *Elymus nutans* dominance and soil N and P
369 fertility (soil PC1 scores; Fig. 4), but not with the soil moisture and organic C gradient
370 (soil PC2 scores; VT composition: $R^2$ = 0.207, $P$ = 0.084; phylogenetic composition:
371 $R^2$ = 0.05, $P$ = 0.574).

372 The NRI of the AM fungal community also declined dramatically across the
373 fertilization gradient ($F_4$ = 12.23, $P$ < 0.001; Fig. 3c) and, it was correlated negatively
374 with soil PC1 scores and *Elymus nutans* dominance, and positively with plant species
375 richness (Table 2); moreover, NRI was negatively correlated with AM fungal genus
376 richness ($r$ = -0.689, $P$ < 0.001) but marginally positively with VT richness ($r$ = 0.392,
377 $P$ = 0.052). The highest NRI occurred in unfertilized control (NRI > 0), middle in F30
378 treatment (NRI = 0), and the lowest in other three high fertilizer treatments (NRI < 0;
379 Fig. 3c). These results indicate that the phylogenetic structure of AM fungal
380 communities were clustered in unfertilized control, random in low level of
381 fertilization (F30), and over-dispersed in high levels of fertilization (F60 to F120).

382

383 **4. Discussion**

384

385 4.1 Fertilization effects on AM fungal richness and community composition
386
387 Negative impacts of fertilization on AM fungal diversity have been widely
388 reported in many ecosystems (e.g. Egerton-Warburton et al., 2007; Alguacil et al.,
389 2010; Camenzind et al., 2014; Chen et al., 2014). Our results corroborate this and
390 provide new evidence showing that *Glomus* species but not the non-*Glomus* species
391 are largely lost by increasing soil N and P fertility in an alpine meadow (Fig. 3 and
392 Table 2). In contrast to our first expectation, surprisingly, genus richness of AM fungi
393 was increased significantly across our fertilization gradient (Fig. 3). Richness at the
394 genus level has rarely been measured, although some studies have shown that AM
395 fungal responses to changing environments might be different at the genus level
396 (Klironomos et al., 1998; Bainard et al., 2014). In our study, the relative abundance of
397 *Glomus* dramatically declined (from 81% in F0 to 14% in F120) while *Diversispora*
398 increased (4.6% to 43%) with increasing soil N and P fertility (Fig. 2); moreover, the
399 highest level of fertilization caused the VT number of *Glomus* to decline by near
400 three-fold (Fig. 3a). Thus, a possible explanation for our observed increase in genus
401 richness is that the loss of dominance by *Glomus* caused by high fertilizer treatments
402 may have provided more opportunities for successful colonization by VTs belonging
403 to other genera.
404 As expected, both species and phylogenetic compositions of our AM fungal
405 communities varied among treatments (Fig. 4). These results are consistent with
406 previous studies in other ecosystems showing significant impacts of N and/or P
407 fertilization on the species composition of AM fungal communities (e.g. Egerton-
408 Warburton et al., 2007; Alguacil et al., 2010; van Diepen et al., 2011; Camenzind et al.,
409 2014), and also corroborate a nearby study (Liu et al., 2015). Interestingly, we found
410 that a low level of fertilization caused significant turnover in species composition but
411 not in phylogenetic composition. This phenomenon was also found in a study of soil
412 bacterial community responses to N fertilization (Fierer et al., 2012), and indicates
413 that the phylogenetic turnover of a given community might be unlikely under slight
414 environmental change. It has been suggested that shifts of AM fungal community
415 structure in a local scale might be related with the changes in environmental
416 conditions and/or competition dynamics of AM fungi (Dumbrell et al., 2010, 2011;
417 Caruso et al., 2012; Maherali and Klironomos, 2012; Horn et al., 2014). This idea is
418 supported by our results showing that both species and phylogenetic compositions

419 were significantly correlated with those plant and soil variables that were very
420 sensitive to fertilizer treatments and the increasing importance of fungal competition
421 across the fertilization gradient (see discussion below). Future studies are necessary to
422 address the ecological consequences of the increasing dominance of VT62 (unknown
423 VT within *Diversispora*) and VT193 (*Claroideoglomus etunicatum* related VT) with
424 fertilization, because high soil fertility may be expected to select AM fungi that form
425 less mutualistic or even parasitic mycorrhiza (Johnson, 1993; Johnson et al., 2015).
426
427 4.2 Fertilization effects on phylogenetic structure of AM fungi and the inferred
428 ecological processes
429
430 We found that AM fungal communities were phylogenetically clustered in
431 unfertilized soil, random in the low fertilizer treatment (F30) and over-dispersed in the
432 other three high fertilizer treatments (Fig. 3c), suggesting that the co-occurring AM
433 fungal taxa were more closely related in unfertilized soil and more distantly related in
434 highly fertilized soil (Webb et al., 2002). Few studies have examined the phylogenetic
435 patterns of soil-derived AM fungal communities, but existing evidence from
436 colonized roots shows that communities are frequently clustered in natural
437 undisturbed ecosystems (e.g. Horn et al., 2014; Saks et al., 2014; Shi et al., 2014; Liu
438 et al., 2015). In fact, AM fungal communities in both roots (data not shown) and soil
439 were phylogenetically clustered in our unfertilized control, suggesting that the
440 phylogenetic structure in root- and soil-derived communities might be similar in
441 natural ecosystems (but see Saks et al., 2014).
442 Because the functional traits of AM fungi are phylogenetically conserved (Powell
443 et al., 2009; Maherali and Klironomos, 2012), we can confidently infer that the
444 primary ecological processes determining AM fungal communities shifted from
445 environmental filtering (in F0) to a stochastic process (F30) and finally to competitive
446 exclusion (F60, F90 and F120) across our fertilization gradient. Our previous
447 observations in this experimental system revealed that both plant allocation to
448 mycorrhiza and plant species richness decreased significantly with increasing soil
449 fertility (Liu et al., 2012). Thus, the shifts of phylogenetic patterns observed in our
450 study are likely to be related to increasing dominance of competition for host plant
451 carbohydrates. The shift of phylogenetic structure in our case was tightly linked with
452 a large loss of *Glomus* species and emergence of other genera. However, whether

*Glomus* species in our study site are weak competitors for carbohydrates or whether they just have very strong host specificity remain to be determined through further investigation. Although competition can sometimes produce a phylogenetically clustered pattern even when functional traits are phylogenetically conserved (Mayfield and Levine, 2010), in our case, a clustered pattern in unfertilized soil can be largely attributed to environmental filtering, because plants under extremely low P availability (<3 mg $kg^{-1}$ soil in our study site) can be expected to actively select AM fungi with high P-uptake ability (Kiers et al., 2011; Bever, 2015), which has been shown to be phylogenetically conserved (Powell et al., 2009).

### 4.3 Comparison of AM fungal taxa detected from soil- and root-derived DNA

Molecular identification of AM fungal communities are usually based on root-derived DNA (e.g. Liu et al., 2011; Öpik et al., 2013; Camenzind et al., 2014; Grilli et al., 2014; Mao et al., 2014), but this method may underestimate AM fungal diversity because of the omission of dormant spores. In the present study, we used soil DNA to identify the AM fungal communities and expected that it may be superior to than root-derived DNA. Our finding of 37 AM fungal VTs is the same as the number detected from mixed roots that were sampled synchronously in the same experimental plots (Liu et al., 2012), although fewer clones were examined in this study (1200 vs. 3600 clones). We can expect that more VTs would be detected from soil than from roots if the same number of clones had been screened. However, comparison of the detected VTs here and in Liu et al. (2012) showed that some dominant VTs in roots (e.g. *Glomus intraradices* related VT) were rarely or never detected in this study, and vice versa (e.g. *Septoglomus constrictum* related VT, Table 1). These differences support previous findings of varying AM fungal taxa identified from root- and soil-derived DNA (e.g. Hempel et al., 2007; Yang et al., 2013; Bainard et al., 2014; Saks et al., 2014) and also suggest that assessment of AM fungal biodiversity might be more accurate using both root- and soil-extracted DNA (Chen et al., 2014).

### 4.4 Conclusions

Our study reveals the compositional changes of AM fungal communities and infers the ecological processes driving these changes across a long-term fertilization

487 gradient in an alpine meadow ecosystem. In our experiment, increasing soil N and P
488 fertility significantly reduced the VT richness (especially the *Glomus* VT richness) but
489 increased genus richness of AM fungi, and the shifts of AM fungal community
490 structure implies an increasing importance of competition among fungi for limited
491 host resources. These findings support our research hypothesis and highlight the
492 contribution of competition to AM fungal community changes under increasing soil
493 fertility. To our best knowledge, this is the first study to analyze phylogenetic
494 structure to explore the ecological processes driving AM fungal responses to an
495 environmental gradient. Given the increasing availability of phylogenetic data,
496 merging phylogenetic information with studies of belowground community ecology
497 should be encouraged in future research to help us more fully understand changes in
498 diversity and to predict the dynamics of biotic communities under changing
499 environments (Cavender-Bares et al., 2009).
500
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510

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512

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**Table**

**Table 1** Taxonomic description of each arbuscular mycorrhizal fungal virtual taxon (VT) and their relative abundances (%, proportion of clone numbers) in different fertilization treatments and the ANOVA summary of the effects of fertilization on each VT (linear mixed effects model). F0, F30, F60, F90 and F120 represent different levels of fertilizer application. Significant effects are in bold.

| | Taxonomic information | | Fertilization treatment | | | | | ANOVA summary | |
|---|---|---|---|---|---|---|---|---|---|
| | Related genus | Related species | F0 | F30 | F60 | F90 | F120 | *F*-value | *P*-value |
| VT222 | *Glomus* | *G. indicum* | 7.3 | 15.5 | 0.6 | – | – | **14.74** | **<0.001** |
| VT125 | *Glomus* | – | 1.2 | – | – | 3.4 | – | **3.34** | **0.036** |
| VT130 | *Glomus* | – | 0.6 | 4 | – | – | 0.6 | 2.44 | 0.090 |
| VT159 | *Glomus* | – | – | 0.6 | 3.6 | – | – | **7.50** | **0.001** |
| VT129 | *Glomus* | – | 0.6 | | | | | 1.00 | 0.436 |
| VT373 | *Glomus* | – | 10.4 | – | – | – | – | **40.40** | **<0.001** |
| VT371 | *Glomus* | – | 27.4 | 8.6 | – | – | – | **19.45** | **<0.001** |
| VT393 | *Glomus* | – | – | 4 | – | 0.6 | – | **7.68** | **0.001** |
| VT172 | *Glomus* | – | – | 2.3 | 2.4 | 1.7 | 1.3 | 0.92 | 0.477 |
| VT137 | *Glomus* | – | 6.1 | 5.7 | – | 3.4 | – | **4.46** | **0.013** |
| VT143 | *Glomus* | – | – | 2.3 | 3.6 | – | – | **4.31** | **0.015** |
| VT135 | *Glomus* | – | 9.8 | – | 8.5 | – | – | **19.30** | **<0.001** |
| VT166 | *Glomus* | – | 15.9 | 1.1 | 8.5 | – | 5.1 | **13.95** | **<0.001** |
| VT199 | *Glomus* | *G. macrocarpum* | 2.4 | 1.1 | 1.8 | 6.9 | – | 2.33 | 0.100 |
| VT174 | *Glomus* | – | – | 0.6 | – | – | – | 1.00 | 0.436 |
| VT304 | *Glomus* | – | – | – | – | – | 1.9 | 2.47 | 0.086 |
| VT151 | *Glomus* | – | – | – | – | – | 5.1 | 2.31 | 0.103 |
| VT214 | new genus 1 | – | – | – | 6.1 | – | – | **9.54** | **<0.001** |
| VT212 | new genus 1 | – | – | – | – | – | 1.9 | 1.91 | 0.158 |
| VT177 | new genus 1 | – | – | – | – | – | 1.9 | 1.00 | 0.436 |
| VT83 | new genus 2 | – | – | 2.3 | – | 0.6 | 0.6 | 1.10 | 0.391 |
| VT325 | *Rhizophagus* | – | – | 16.1 | 17 | 16.6 | 3.2 | **5.12** | **0.008** |
| VT113 | *Rhizophagus* | *R. intraradices* | 0.6 | – | 0.6 | – | – | 0.76 | 0.566 |
| VT295 | *Rhizophagus* | – | 0.6 | – | – | – | – | 1.00 | 0.436 |
| VT64 | *Septoglomus* | *S. constrictum* | – | 10.9 | 2.4 | 28 | 3.2 | **7.73** | **0.001** |
| VT65 | *Funneliformis* | *F. caledonium* | 0.6 | 0.6 | – | – | 2.5 | 2.71 | 0.068 |
| VT67 | *Funneliformis* | *F. mosseae* | – | – | – | 0.6 | 4.5 | **3.61** | **0.028** |
| VT356 | *Redeckera* | – | – | – | 0.6 | 2.3 | – | 1.76 | 0.187 |
| VT380 | *Diversispora* | – | – | 0.6 | – | – | – | 1.00 | 0.436 |
| VT60 | *Diversispora* | *D. celata* | – | – | 5.5 | – | – | **3.32** | **0.037** |
| VT54 | *Diversispora* | *D. aurantia* | 4.3 | – | – | – | – | **5.81** | **0.004** |
| VT62 | *Diversispora* | – | – | 5.2 | 17 | 13.7 | 39.5 | **17.64** | **<0.001** |
| VT56 | *Claroideoglomus* | – | 1.2 | 6.9 | 21.8 | 10.9 | – | **10.24** | **<0.001** |
| VT57 | *Claroideoglomus* | – | – | 11.5 | – | 10.9 | – | **5.58** | **0.005** |
| VT193 | *Claroideoglomus* | *C. etunicatum* | 8.5 | – | – | – | 28.7 | **11.97** | **<0.001** |
| VT245 | *Archaeospora* | *Ar. trappei* | – | – | – | 0.6 | – | 1.00 | 0.436 |
| VT283 | *Ambispora* | *Am. fennica* | 2.4 | – | – | – | – | **4.46** | **0.013** |

6 **Table 2** Correlations between virtual taxon (VT) richness, genus richness and the nearest relative index (NRI) of arbuscular mycorrhizal fungal
7 community with two PCA extracted components of soil properties (soil PC1 and PC2), plant species richness and the relative abundance of
8 *Elymus nutans*. Soil PC1 reflects positively the soil N and P fertility gradient; soil PC2 reflects positively the soil moisture and organic C
9 gradient. Values are Pearson's correlation coefficients. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. Significant correlations are in bold.
10

| | Soil PC1 | Soil PC2 | Plant species richness | Abundance of *Elymus nutans* |
|---|---|---|---|---|
| VT richness of all lineages | **-0.53**** | -0.05 | 0.38 | **-0.57**** |
| VT richness of *Glomus* | **-0.79***** | -0.24 | **0.77***** | **-0.82***** |
| Genus richness | **0.44*** | 0.23 | **-0.64***** | **0.52**** |
| NRI | **-0.69***** | -0.30 | **0.76***** | **-0.65***** |

11

1 **Figure captions**

2

3 **Fig. 1** Maximum likelihood phylogenetic tree of representative sequences of each
4 arbuscular mycorrhizal fungal virtual taxon (VT371 etc.) obtained in this study and
5 referenced sequences from the GenBank database. Arbuscular mycorrhizal fungal VTs
6 were delimited according to the sequence similarity (≥97%), query coverage (≥97%)
7 and BLAST bit score after BLAST against the online Maarj*AM* database. The
8 nomenclatures of arbuscular mycorrhizal fungal genera and families are according to
9 Schüßler and Walker, 2010 (http://schuessler.userweb.mwn.de/amphylo/).

10

11 **Fig. 2** Linear regressions of the relative abundance of *Glomus* and *Diversispora*
12 versus the two PCA extracted components of soil properties (PC1 and PC2), relative
13 abundance of *Elymus nutans* and plant species richness.

14

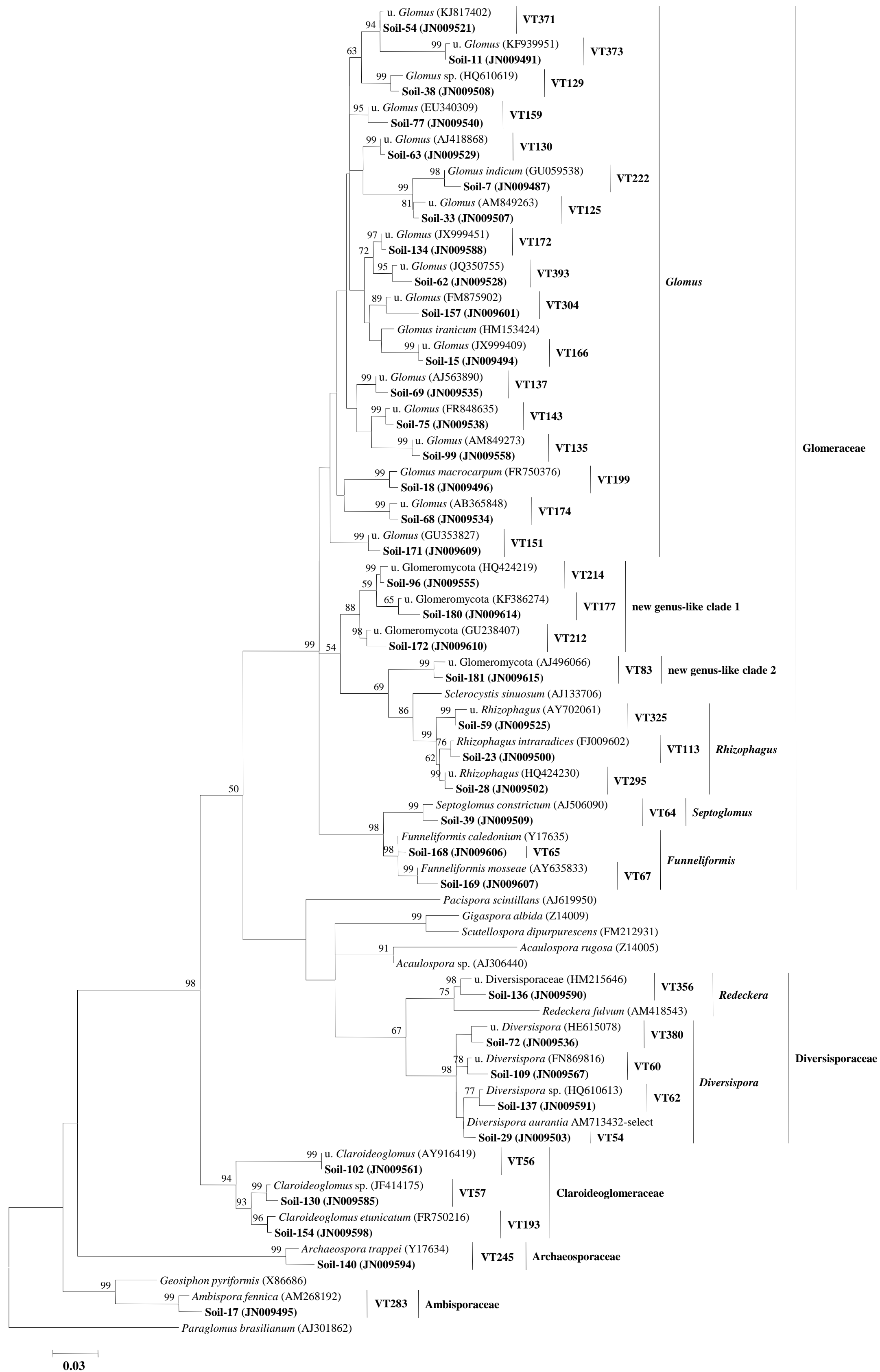
15 **Fig. 3** The virtual taxon (VT) richness, genus richness and the nearest relative index
16 (NRI) of arbuscular mycorrhizal fungal community varied across a fertilization
17 gradient. Bars represent means ± SEs ($n$ = 5). F0, F30, F60, F90 and F120 represent
18 different levels of fertilizer application. For (a) and (b), the significant differences
19 between columns are indicated with dissimilar letters using Tukey's HSD test ($P \leq$
20 0.05) after fitting linear mixed effects models. For (c), asterisk indicates that the NRI
21 is significantly different from zero after $T$-test (ns, non–significant; * $P \leq 0.05$; ** $P \leq$
22 0.01).

23

24 **Fig. 4** Non–metric multidimensional scaling (NMDS) patterns of arbuscular
25 mycorrhizal fungal community dissimilarities among fertilization treatments using the
26 data of virtual taxon composition (a) and phylogenetic composition (betaMPD; b).
27 Ellipses with different colors indicate 95% confidence ellipses for each treatment.
28 Correlations between each community ordination with the two PCA extracted
29 components of soil properties (PC1 and PC2), relative abundance of *Elymus nutans*
30 and plant species richness were jointed and the significant vectors ($P$ < 0.05) were
31 shown. F0, F30, F60, F90 and F120 represent different levels of fertilizer application.

32

33

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35

36 **Fig. 1**

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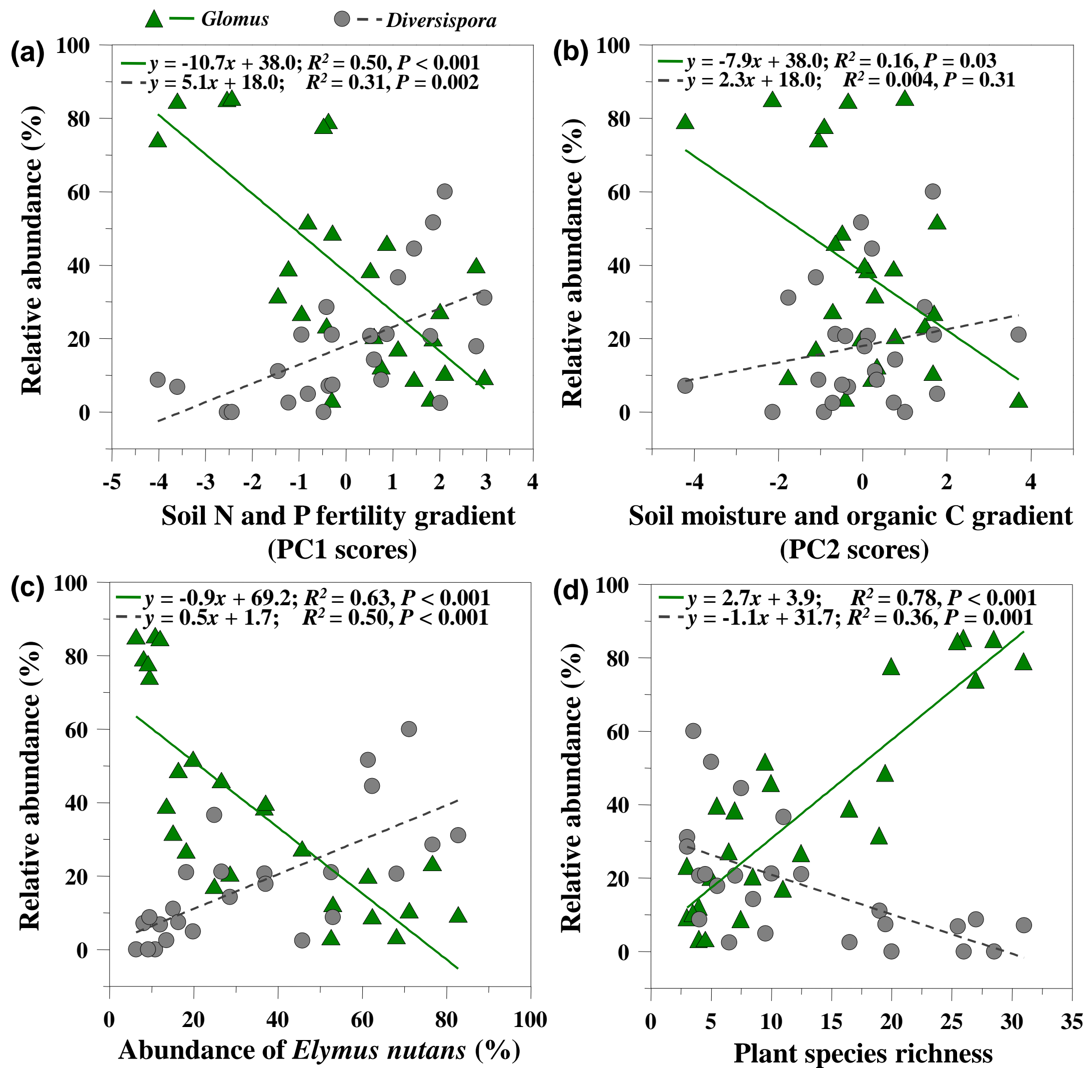
38

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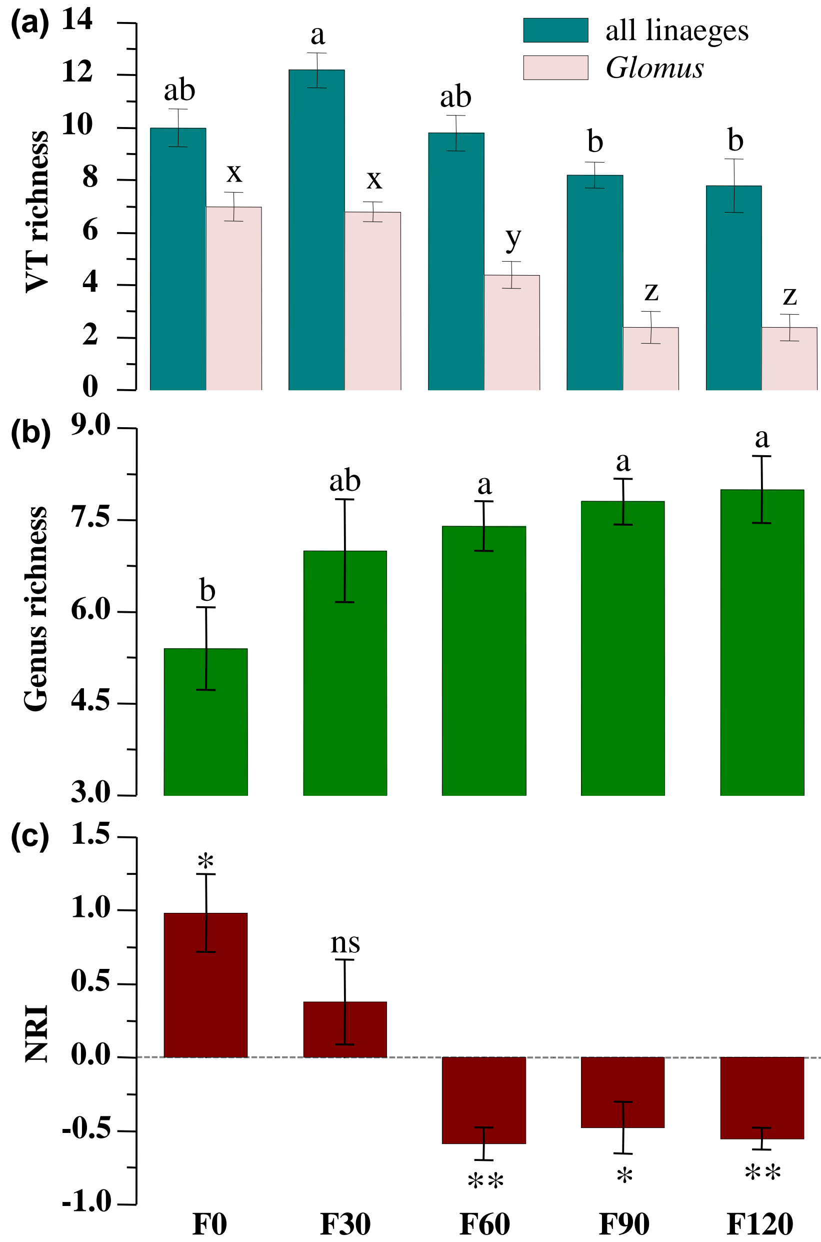
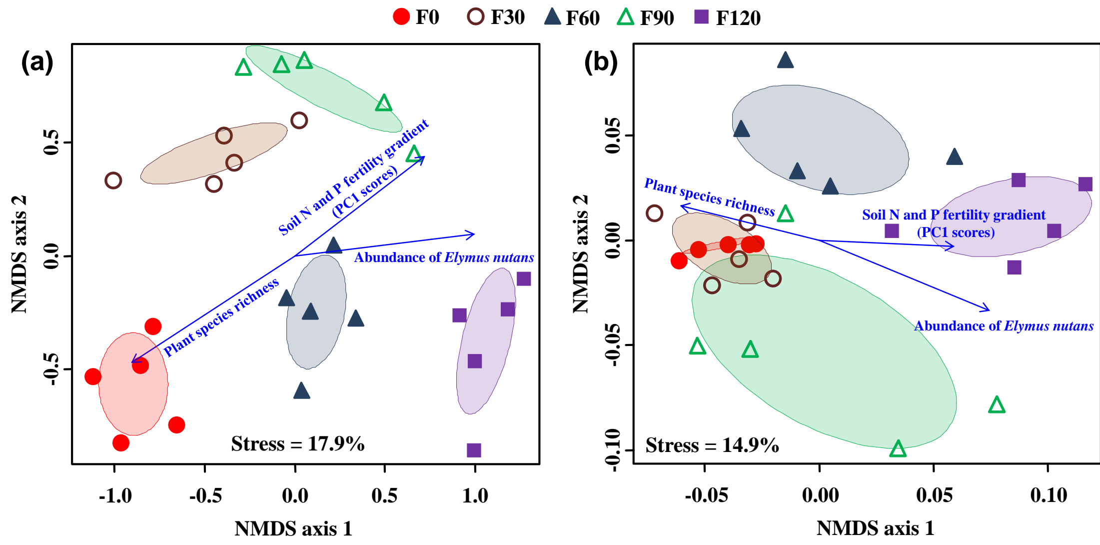
40

41 **Fig. 2**




42
43
44 **Fig. 3**

45

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47

48 **Fig. 4**

49