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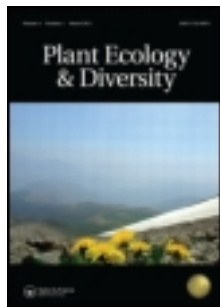
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Basin-wide variations in Amazon forest nitrogen-cycling characteristics as inferred from plant and soil $^{15}\text{N}/^{14}\text{N}$ measurements

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1 **Basin-wide variations in Amazon forest nitrogen-cycling characteristics as inferred**
2 **from plant and soil ¹⁵N/¹⁴N measurements**

3

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24

25

26 **Abstract**

27 **Background:** Patterns in tropical forest nitrogen cycling are poorly understood. In
28 particular, the extent to which leguminous trees in these forests fix nitrogen is unclear.

29 **Aims:** Determine factors that explain variation in foliar $\delta^{15}\text{N}$ ($\delta^{15}\text{N}_\text{F}$) for Amazon forest
30 trees. Evaluate extent to which putatively N_2 -fixing Fabaceae acquire nitrogen from the
31 atmosphere.

32 **Methods:** Upper-canopy $\delta^{15}\text{N}_\text{F}$ were determined for 1255 trees sampled across 65
33 Amazon forest plots. Along with plot inventory data, differences in $\delta^{15}\text{N}_\text{F}$ between
34 nodule-forming Fabaceae and other trees were used to estimate the extent of N_2 -
35 fixation.

36 **Results:** $\delta^{15}\text{N}_\text{F}$ ranged from -12.1‰ to $+9.3\text{‰}$. Most of this variation was attributable to
37 site-specific conditions with extractable soil phosphorus and dry season precipitation
38 having strong influences, suggesting a restricted availability of nitrogen on both young
39 and old soils and/or at low precipitation. Fabaceae constituted fewer than 10% of the
40 sampled trees, and only 36% were expressed fixers. We estimated an average Amazon
41 forest symbiotic fixation rate of $3 \text{ kg N ha}^{-1}\text{year}^{-1}$.

42 **Conclusion:** Plant $\delta^{15}\text{N}$ indicate that low levels of nitrogen availability are only likely
43 to influence Amazon forest function on immature or old weathered soils and/or where
44 dry season precipitation is low. Most Fabaceae species that are capable of nodulating do
45 not fix nitrogen in Amazonia.

46 **Key words:** ^{15}N natural abundance; Amazon, Fabaceae, nitrogen; nitrogen fixation,
47 phosphorus; tropical forest

48

49 **Introduction**

50 Tropical forests play a key role in the global nitrogen cycle with their vegetation
51 typically rapidly cycling nitrogen through growth and litterfall processes (Nye 1960;
52 Vitousek 1984; Vitousek and Sanford 1986; Lloyd et al. 2009). Tropical forest soils are
53 also characterised by relatively high rates of mineralisation and nitrification as
54 compared to their temperate counterparts (Silver et al. 2000; 2005; Pardo et al. 2006).
55 They can also sustain relatively high rates of loss of N₂O to the atmosphere (Davidson
56 et al. 2004; Keller et al. 2005; Houlton et al. 2006) as well as release substantial
57 amounts of nitrogen through riverine export (Lewis et al. 1999; Hedin et al. 2003;
58 2009).

59 Tropical forests are often characterised by a high abundance of members of the
60 family Fabaceae (Gentry 1988), principally among large to medium-sized trees and
61 lianas. Some species of this family live in symbiosis with rhizobia that are able to fix
62 nitrogen from the air and provide it to the host plant in exchange for carbohydrates
63 (Sylvester-Bradley et al. 1980; Salati et al. 1982; De Faria et al. 1989; Moreira et al.
64 1992; McKey 1994; Sprent 1994, 1995, 1996; Vitousek et al. 2002; Pons et al. 2007;
65 Hedin et al. 2009; Barron et al. 2011). Several authors have hypothesised that an
66 apparent nitrogen-richness of tropical forests can be attributed to these N₂-fixing trees
67 (Jenny et al. 1948, 1949; Roggy, Prevost, Garbaye, et al. 1999; Pons et al. 2007).
68 Nevertheless, some studies have shown Fabaceae in undisturbed mature tropical forests
69 to not fix nitrogen, even though the genera involved clearly had the ability to do so. It
70 has thus been argued that N₂-fixation may only occur in cases of transient nitrogen
71 limitation (Gehring et al. 2005; Ometto et al. 2006; Barron et al. 2011). Hedin et al.
72 (2009) have termed the apparent contradiction of a high abundance of potentially N₂-
73 fixing Fabaceae in tropical forests despite the apparent over-abundance of nitrogen in

74 most tropical forest stands as the “nitrogen paradox” of tropical forests. Despite the
75 potential importance of N₂-fixing Fabaceae in influencing the nitrogen cycle of tropical
76 forests there have been surprisingly few estimates of symbiotic N₂-fixation by these
77 forests. In the lowland tropical forests of Guiana estimates ranged from 4 to 7 kg ha⁻¹
78 year⁻¹ (Roggy, Prevost, Garbaye, et al. 1999; Pons et al. 2007). Based on a combined
79 data/modelling exercise, Cleveland et al. (2010) found the same range of symbiotic
80 fixation in the south-west Brazilian Amazon region. These estimates are much lower
81 than a global average estimate of symbiotic fixation for tropical forests of 16 kg ha⁻¹
82 year⁻¹, and ca. 20 to 25 kg ha⁻¹ year⁻¹ estimated by Cleveland et al. (1999) and Houlton
83 et al. (2008), respectively. Due to this large range of estimates and their uncertainties,
84 there is a clear need for further investigations on this topic.

85 From both an observational and theoretical viewpoint, it is expected that relatively
86 high losses of nitrogen (a ‘leaky system’) should leave soils and vegetation enriched in
87 ¹⁵N in relation to ¹⁴N (Högberg 1997). With $\delta^{15}\text{N}$ inputs via nitrogen biological fixation
88 or through wet and/or dry deposition having a $\delta^{15}\text{N}$ of ~0‰ (Hoering and Ford 1960;
89 Amarger et al. 1977; Delwiche et al. 1979; Högberg 1986; Shearer and Kohl 1986;
90 Peoples and Craswell 1992; Sprent et al. 1996; Roggy, Prevost, Garbaye, et al. 1999;
91 Gehring et al. 2005; Pons et al. 2007), soil and vegetation systems with relatively high
92 nitrogen losses thus tend to have higher $\delta^{15}\text{N}$ values than those where the nitrogen cycle
93 is more closed (i.e., with relatively low nitrogen loss). As a result of this, plant and soil
94 $\delta^{15}\text{N}$ in nitrogen-rich tropical forests are higher and predicted to be less variable than
95 nitrogen-poor temperate forests (Nadelhoffer et al. 1996; Martinelli et al. 1999).
96 Measurements of the ¹⁵N:¹⁴N ratio of soils and vegetation can thus provide a first
97 approximation of relative nitrogen abundance within an ecosystem and the associated

98 relative openness of the nitrogen cycle (Vitousek et al. 1989; Martinelli et al. 1999;
99 Houlton et al. 2006; Pardo et al. 2006; Craine et al. 2009; Posada and Schuur 2011).

100 Any symbiotic nitrogen fixation by Fabaceae–rhizobium associations also has the
101 capability to influence the $\delta^{15}\text{N}$ of plant material. This is because, with a $\delta^{15}\text{N}$ of ca. 0‰
102 the $^{15}\text{N}:^{14}\text{N}$ ratio of atmospheric nitrogen is generally lower than that of the soil nitrogen
103 pool, the latter usually being considered the most important source of nitrogen to plants
104 (Hoering and Ford 1960; Delwiche et al. 1979). This difference in $^{15}\text{N}:^{14}\text{N}$ ratios
105 between the two nitrogen sources also allows the relative reliance of the Fabaceae on N_2
106 fixation to be estimated by comparing the foliar stable nitrogen isotopic composition of
107 the foliage ($\delta^{15}\text{N}_\text{F}$) of the N_2 -fixing legume to a non- N_2 -fixing reference plant (Shearer
108 and Kohl 1986; Unkovich et al. 2008).

109 Among tropical forests, the Amazon is the largest continuous tropical biome with
110 ca. 6 million km^2 . It is of pivotal importance globally due to its high biodiversity, soil
111 and biomass stocks of carbon and nitrogen, and capability to influence the global
112 climate due to the huge amount of water and energy that is recycled through the
113 vegetation of this region. Although sometimes viewed as a single tract of tropical forest,
114 this region encompasses distinct climates, parent material, soils and vegetation. For
115 example, Fyllas et al. (2009) recently showed a large variability in leaf chemical
116 composition, leaf mass:area ratios and $\delta^{13}\text{C}$ composition for trees sampled in about 60
117 plots across the Amazon region. They further found that some leaf nutrients were
118 mostly linked to the taxonomic affiliation, while others were more closely associated
119 with soil chemical composition and/or rainfall regime. Similar results were found by
120 Patiño et al. (2009) examining Basin-wide variations in branch xylem density, as well as
121 variations in a range of tree physical traits, such as leaf size, leaf area:sapwood area
122 ratio and species maximum height (Patiño, Lloyd, Paiva, et al. 2012).

123 Investigating the physical and chemical characteristics of soils sampled across the
124 same plots as Fyllas et al. (2009) and Patiño et al. (2009), Quesada et al. (2010) found
125 an interesting relationship between the soil stable nitrogen isotopic composition ($\delta^{15}\text{N}_\text{S}$)
126 and soil total extractable phosphorus concentration, both integrated from the soil surface
127 to 0.3 m depth, the latter being denoted here as $[\text{P}]_\text{ex}$. They found a positive correlation
128 of $[\text{P}]_\text{ex}$ with $\delta^{15}\text{N}_\text{S}$ up to about $[\text{P}]_\text{ex} = 30 \text{ mg kg}^{-1}$. However, beyond this point $\delta^{15}\text{N}_\text{S}$
129 started to slowly decline with increasing $[\text{P}]_\text{ex}$. The lowest $\delta^{15}\text{N}_\text{S}$ values were thus found
130 in sandy soils with low fertility and low $[\text{P}]_\text{ex}$ with the highest $\delta^{15}\text{N}_\text{S}$ on older soils, e.g.,
131 Ferralsols and Acrisols. Because of the decline at high $[\text{P}]_\text{ex}$, the youngest soils, e.g.,
132 Cambisols and Alisols, had systematically lower $\delta^{15}\text{N}_\text{S}$ than the more mature soils (see
133 Figure S1). Thus the relationship between $[\text{P}]_\text{ex}$ and $\delta^{15}\text{N}_\text{S}$ was found to be distinctly
134 non-linear.

135 It has also been reported that the $\delta^{15}\text{N}_\text{S}$ and $\delta^{15}\text{N}_\text{F}$ of Amazon forest may be
136 influenced by soil texture with $\delta^{15}\text{N}$ of both leaf and soil higher in clay than in sandy
137 soils (Silver et al. 2000; Nardoto et al. 2008; Sotta et al. 2008; Mardegan et al. 2009).
138 Authors working in other regions have observed $\delta^{15}\text{N}_\text{F}$ to decrease with increased
139 precipitation (Heaton 1987; Schulze et al. 1998; Austin and Vitousek 1998; Handley et
140 al. 1999; Amundson et al. 2003; Craine et al. 2009; Posada and Schuur 2011) and it has
141 similarly been suggested that precipitation influences $\delta^{15}\text{N}_\text{F}$ for the forests of the
142 Brazilian Amazon region as trees growing on wetter sites tend to have lower $\delta^{15}\text{N}_\text{F}$ than
143 those at relatively drier sites (Nardoto et al. 2008). This influence of soil texture and
144 precipitation on the $\delta^{15}\text{N}_\text{F}$ for Amazon forest trees has, however, only been
145 demonstrated for a few forests, mostly growing on old mature soils (Silver et al. 2000;
146 Nardoto et al. 2008; Sotta et al. 2008). By contrast, the relationship between soil $[\text{P}]_\text{ex}$
147 and $\delta^{15}\text{N}_\text{S}$ demonstrated by Quesada et al. (2010) encompassed more than 60 sample

148 sites across the Amazon Basin with different parent materials, soils, and precipitation
149 regimes.

150 In this study we analysed $\delta^{15}\text{N}_F$ for 1255 trees across 65 Amazon forest plots,
151 from 62 botanical families, 241 genera, and more than 400 species. Soil types of the
152 study plots were diverse, including white sands (Arenosols or Podzols), older tropical
153 soils, such as Ferralsols and Acrisols - often considered more typical of tropical forests
154 soils - to younger less weathered soils such as Cambisols (generally close to the Andes),
155 with $[\text{P}]_{\text{ex}}$ varying from a minimum of ca. 20 mg kg⁻¹ in old sandy soils to ca. 400 mg
156 kg⁻¹ in some younger soils.

157 We were specifically interested to ascertain what factors would explain the
158 variability in $\delta^{15}\text{N}_S$ observed. Specifically:

159 a) Is the relationship between $\delta^{15}\text{N}_S$ and $[\text{P}]_{\text{ex}}$ reflected in large scale $\delta^{15}\text{N}_F$
160 patterns and to what extent do the potentially N₂-fixing Fabaceae of the lowland tropical
161 forests of the Amazon Basin fix atmospheric nitrogen?

162 b) Is there an effect of precipitation gradients as well as soil age gradient across
163 the Amazon Basin on $\delta^{15}\text{N}_F$ as observed for other ecosystems?

164

165 **Materials and methods**

166 *Study area*

167 A total of 65 primary forest plots across the Amazon Basin (see Fyllas et al. 2009 and
168 Quesada et al. 2010 for more details) were selected from the RAINFOR
169 (<http://www.rainfor.org>) and LBA projects (<http://www.lbaeco.org/>). We also included
170 seven extra plots towards the southern forest margin added from the 'Tropical Biomes
171 in Transition' (TROBIT) project (Torello-Raventos et al. 2013) for which both soil and
172 foliar nutrient and isotope data were introduced here for the first time (Table S1). Taken

173 together, the plots sampled encompassed the forest vegetation of six different countries
174 (Bolivia, Brazil, Colombia, Ecuador, Peru and Venezuela) with a wide variety of soil
175 type and climatic conditions (see Table S2; Fyllas et al. 2009; Quesada et al. 2010;
176 Torello-Raventos et al. 2013 for more details).

177 Our survey included significant areas outside the Amazon watershed, in
178 particular, areas of the Orinoco Basin, the Guyana lowlands in Venezuela in the north
179 and the Bolivian and Brazilian peripheral areas at the southern edge of the forest
180 margin. These areas do, however, form a phytogeographic continuum with Amazon
181 lowland rainforest. Hence it is reasonable to adopt the shorthand 'Amazonia' to describe
182 this entire lowland tropical forest region. For the dataset described in Torello-Raventos
183 et al. (2013) which encompasses the forest-savanna transition zone of central-southern
184 Brazil and north-eastern Bolivia, only stands identified as consisting predominantly of
185 species usually found in forest vegetation were included; this excluded stands
186 dominated by species usually associated with savanna, even when growing in close
187 proximity to, and/or of a similar structure to, nearby forest formations. All plots were
188 situated in areas ranging from 20 m to 700 m above sea level, with mean annual
189 temperature varying from approximately 23°C to 27°C, and a mean precipitation
190 ranging from 820 mm to 4110 mm year⁻¹.

191

192 *Laboratory analyses*

193 Data for nitrogen stable isotope ratios as well as for foliar nitrogen and phosphorus
194 concentrations were obtained at individual plant level. A total of 1255 $\delta^{15}\text{N}_F$ values
195 were used in this analysis, representing ca. 20 randomly chosen trees per site. Data for
196 foliar nitrogen and phosphorus concentrations were obtained for upper canopy leaves as
197 described in Lloyd et al. (2010). The $\delta^{15}\text{N}_S$ and phosphorus availability in the surface

198 soil (0-30 cm) were determined as described in Quesada et al. (2010). Briefly, total
199 extractable phosphorus, $[P]_{\text{ex}}$ was obtained from a modified phosphorus fractionation
200 scheme (Hedley et al. 1982), where different phosphorus pools are extracted
201 sequentially with $[P]_{\text{ex}}$ considered to represent an amalgamation of the different forms
202 of soil phosphorus potentially available to plants. An index of soil physical properties
203 (Π_1) proposed by Quesada et al. (2010) was also utilised here (Figure S2). This
204 represents a measure of soil physical properties that can potentially limit plant growth
205 and establishment, such as effective soil depth, soil structure, topography and extent of
206 anoxic conditions.

207 For the Brazilian, Colombian and Venezuelan samples $\delta^{15}\text{N}_\text{S}$ and $\delta^{15}\text{N}_\text{F}$ were
208 analysed by using an isotope ratio mass spectrometer at Centro de Energia Nuclear na
209 Agricultura (CENA) in Piracicaba, Brazil, while the Max-Planck Institute für
210 Biogeochemie (MPI) in Jena, Germany analysed samples from Ecuador and Peru and
211 most sites in Bolivia. Soil material from some Bolivian sites sampled as part of the
212 TROBIT project (plots ACU-01, TUC-01, OTT-01, LFB-02) were analysed at the
213 University of St. Andrews, Scotland, with leaf material analysed for $\delta^{15}\text{N}$ at James Cook
214 University, Australia. Laboratories were under inter-calibration exercises and used same
215 methodologies and sample standards and we estimate an overall precision of $\pm 0.2\%$.

216

217 *Climatological data*

218 Mean annual temperature (T_A), mean annual precipitation (P_A) and the mean monthly
219 precipitation for the driest quarter (P_D) were derived from interpolated grid cells from
220 the WorldClim database (<http://www.worldclim.org/>; see Fyllas et al. 2009 for more
221 details).

222

223 *Floristic analyses*

224 The proportional abundance of potentially N₂-fixing Fabaceae and those considered
225 incapable of nodulation (ϕ_F and ϕ_{NF} respectively) was evaluated for each study plot
226 (usually 1 ha) using tree-by-tree data from the RAINFOR/ForestPlots.net database
227 (Lopez-Gonzalez et al. 2011; Patiño, Lloyd, Loyd, et al. (2012). These calculations were
228 made on a stem number per species basis and therefore ignore any systematic
229 differences in tree size between the two different nodulating classes of the Fabaceae as
230 well as any systematic differences with trees from other families in the same stand.

231

232 *N₂-fixation status during survey*

233 Recent N₂-fixation by potentially nodulating Fabaceae species was assumed to have had
234 occurred when the $\delta^{15}N$ difference between the individual leaf $\delta^{15}N_F$ and the average
235 $\delta^{15}N_F$ in the same plot ($\delta^{15}N_P$) was >1‰. This is clearly an arbitrary criterion that is if
236 anything too liberal: requiring that only ca. 25% of the nitrogen in a leaf to have come
237 from atmospheric N₂ fixation in order for the tree involved to be declared ‘fixing’ at the
238 typical stand-level $\delta^{15}N_F$ of 4‰. We also checked for ‘leakage’ in this criterion. We
239 therefore considered separately the percentage of the Fabaceae species in our survey
240 known not to be capable to nodulate and would be incorrectly inferred to have recently
241 been ‘N-fixing’ when subject to the same >1‰ criterion.

242

243 *Statistical analysis*

244 We first tested for normality and homogeneity of variance for $\delta^{15}N_F$ and correlated
245 foliar nutrients (nitrogen and phosphorus on dry weight basis). As these properties did
246 not follow a normal distribution, they were transformed using Box-Cox methods.
247 Negative $\delta^{15}N_F$ values were transformed into positive values by incorporating into every

248 $\delta^{15}\text{N}_F$ value the most negative $\delta^{15}\text{N}_F$ value reported (-12.1‰). In order to partition the
249 variance in $\delta^{15}\text{N}_S$ we used the model as proposed by Fyllas et al. (2009):

$$250 \quad \delta^{15}\text{N}_F = \mu + p + f/g/s + \varepsilon, \quad (\text{Equation 1})$$

251 where μ is the overall mean $\delta^{15}\text{N}_F$ of the dataset, p is the plot characteristic (soil and
252 climate), $f/g/s$ represents the genetic structure of the data, i.e. that each individual
253 belongs to a species (s), nested in a genus (g), nested in a family (f), and ε is the
254 residual. The variance of the model was tested by Mixed ANOVA analysis assuming p
255 and $f/g/s$ as random variables in a hierarchically nested design. Residual Maximum
256 Likelihood (REML) was used to estimate model parameters using the STATISTICA
257 software package (Statsoft Inc. 2007). Differences between different Fabaceae groups
258 (non-fixers; not N-fixing and N-fixing during survey) were tested by one-way ANOVA.
259 The non-parametric Spearman correlation coefficient (ρ) was used to test correlations
260 between leaf properties and soil and environmental variables. An adjustment of the
261 relevant degrees of freedom and associated P values was carried out to account for
262 spatial autocorrelation (Dutilleul 1993). We used the software 'Spatial Analysis in
263 Macroecology – SAM' (Rangel et al. 2006), a non-parametric approach that allowed an
264 evaluation of the effects of climate and soils on $\delta^{15}\text{N}_F$ without resort to predefined
265 assumptions as to the exact nature of any simple correlative relationships occurring.

266

267 **Results**

268 From tree-by-tree census data for all 65 plots examined, we found a mean stem fraction
269 of N_2 -fixing (nodulating) Fabaceae, $\varphi_F = 0.08$ and non-nodulating Fabaceae, $\varphi_{NF} = 0.05$.
270 Thus, according to our dataset, potential N_2 -fixing Fabaceae constitute fewer than 10%
271 of the trees of the Amazon forest; and, those members of the Fabaceae considered
272 incapable of nodulation and hence unable to fix N_2 just slightly less abundant as

273 putative N₂ fixers (Table S1). In several cases, such as the Podzol SCR-04 (Venezuela)
274 or the Ferralsol VCR-01 (southern forest-savanna transition zone, Brazil) ϕ_F was <0.01.
275 The highest ϕ_F recorded was 0.26 from a Plinthosol (DOI-02, south-east Amazon, Acre,
276 Brazil). Only two other sites had $\phi_F > 0.2$: a semi-deciduous forest on an Acrisol in the
277 southern forest-savanna transition zone (OTT-01, Bolivia) and a high rainfall forest
278 located on a poorly drained Gleysol (TIP-03, Ecuador). The proportion of non-N₂ fixing
279 Fabaceae also varied widely, being < 0.01 on many sites, but > 0.50 at OTT-01 and for
280 one plot in eastern Venezuela (ELD-12).

281

282 *Variability in foliar $\delta^{15}\text{N}$ ($\delta^{15}\text{N}_F$)*

283 Foliar stable nitrogen isotopic composition varied from -12.1‰ for a *Micrandra*
284 *sprucei* tree growing on a Podzol at San Carlos do Rio Negro (SCR-04, Venezuela) to
285 $+9.3\text{‰}$ for an unidentified *Inga* species on a Ferralsol in eastern Amazonia (TAP-123);
286 this giving an overall range of ca. 21‰ (Figure 1). The mean $\delta^{15}\text{N}_F$ (\pm standard
287 deviation) was equal to $+3.1 \pm 2.3\text{‰}$ ($n=1255$), which was similar to the median value of
288 $+3.2\text{‰}$ (lower quartile = $+1.9\text{‰}$, upper quartile = $+4.6\text{‰}$) (Figure 1). The lowest plot
289 level mean value of $\delta^{15}\text{N}_F$ was observed for SCR-04 ($\delta^{15}\text{N}_P = -4.3\text{‰}$) with the nearby
290 SCR-05 (-0.8‰), and another pair of Podzol plots near Manaus in the central Amazon
291 (MAN-3 and MAN-4) located in ‘baixios’, areas frequently exposed to flooding also
292 having relatively low values for $\delta^{15}\text{N}_P$ of $+0.2\text{‰}$ and -1.4‰ respectively (Table S1). All
293 these soils have very high sand content (Table S1). Highest $\delta^{15}\text{N}_P$ were observed for
294 stands growing on Ferralsols of the eastern Amazon region, in the National Forest of the
295 Tapajós (TAP-123 and TAP-04; $\delta^{15}\text{N}_P = +6.5\text{‰}$ and $+6.2\text{‰}$ respectively), and in the
296 National Forest of Caxiuana to the east (CAX-06; $\langle \delta^{15}\text{N} \rangle = +6.2\text{‰}$). Ferralsols in
297 these areas are characterised by high clay content (Table S1). A high $\delta^{15}\text{N}_P$ was also

298 observed for a deciduous forest on a Cambisol in Bolivia, the lowest precipitation site in
299 the dataset (TUC-01; $\delta^{15}\text{N}_p = +6.8\text{‰}$).

300 From the analysis of Equation 1, differences in $\delta^{15}\text{N}_F$ were mainly attributable to
301 growing location with 0.71 of the variation attributable to plot followed by the residual
302 term (0.22) which represents within-species variation and sampling/measurement error.
303 Genetic characteristics were responsible for only 0.07 of the variation observed with the
304 following distribution: family (0.03), genus (0.02), and species (0.02).

305

306 *Plot climate and soil characteristics and $\delta^{15}\text{N}_p$*

307 As bulk $\delta^{15}\text{N}_S$ does not necessarily reflect the isotopic composition of inorganic forms
308 of nitrogen taken up by plants and with any association of plants with ectomycorrhiza
309 fungi providing plants with nitrogen depleted in ^{15}N (Hobbie et al. 2005; Craine et al.
310 2009), we first tested if the stand level $\delta^{15}\text{N}_p$ reflected that of the surface soil (0.0 - 0.3
311 m). This showed a significant correlation between $\delta^{15}\text{N}_S$ and $\delta^{15}\text{N}_p$ with 70% of the
312 variation in $\delta^{15}\text{N}_p$ accounted for by the soil $^{15}\text{N}:^{14}\text{N}$ ratio (Figure 2a). We also found an
313 inverse relation between $\delta^{15}\text{N}_p$ and P_A , however, only 10% of the variance in $\delta^{15}\text{N}_p$
314 could be explained by P_A alone (Figure 2b).

315 Soils with high sand content (Φ_S) usually have lower mineralisation and
316 nitrification rates that in turn lead to a lower nitrogen availability, decreasing the $\delta^{15}\text{N}_F$
317 at such sites. Based on the trends observed from previous studies in the Amazon region
318 (Silver et al. 2000; Nardoto et al. 2008; Mardegan et al. 2009) we thus tested if $\delta^{15}\text{N}_F$
319 decreased with increasing Φ_S across our dataset. Although a significant negative
320 correlation between $\delta^{15}\text{N}_p$ and Φ_S was found, only 9% of the variance could be
321 explained by Φ_S alone (Figure 2c).

322 Using dry-season precipitation regime to broadly segregate plots into those that
323 usually experience some sort of soil water deficit during the dry season and those which
324 do not - $P_D < 100 \text{ mm month}^{-1}$ and $P_D > 100 \text{ mm month}^{-1}$. This mean monthly
325 precipitation roughly reflects the mean monthly evaporative demand of Amazon forest
326 (Malhi and Wright 2004), we found that for plots with $P_D < 100 \text{ mm}$ the relationship
327 between $[P]_{\text{ex}}$ and $\delta^{15}\text{N}_P$ was similar to that observed for $[P]_{\text{ex}}$ and $\delta^{15}\text{N}_S$ by Quesada et
328 al. (2010; see Figure S1), with lower values at both low and high $[P]_{\text{ex}}$ than at
329 intermediate $[P]_{\text{ex}}$. The Spearman's measure of association (which assumes a monotonic
330 relationship) was not significant ($\rho = 0.36$, $P = 0.25$: Figure 3a). By contrast, there was a
331 significant direct association between $\delta^{15}\text{N}_P$ and $[P]_{\text{ex}}$ for plots with $P_D > 100 \text{ mm}$ ($\rho =$
332 0.45 , $P = 0.03$: Figure 3b). It was also observed that $\delta^{15}\text{N}_P$ for plots with $P_D < 100 \text{ mm}$
333 showed a significantly higher, association with Φ_S than for plots with $P_D > 100 \text{ mm}$ ($\rho =$
334 -0.55 and -0.04 respectively; data not shown).

335 In order to further probe contrasting patterns in the relationships between $\delta^{15}\text{N}_P$
336 and $[P]_{\text{ex}}$ as dependent on dry-season precipitation regime, we investigated variations in
337 stand-level average foliar dry-weight based nitrogen and phosphorus concentrations
338 (denoted as $[N]_{\text{DW}}$ and $[P]_{\text{DW}}$ respectively). There was no difference in $[N]_{\text{DW}}$ ($F_{(1,57)} =$
339 1.03 , $P = 0.31$), but in the plots with $P_D < 100 \text{ mm}$ $[P]_{\text{DW}}$ was significantly lower than in
340 the plots with $P_D > 100 \text{ mm}$ ($F_{(1,57)} = 9.2$, $P < 0.004$). Although there was no significant
341 association between $[N]_{\text{DW}}$ and $[P]_{\text{ex}}$ for plots with $P_D < 100 \text{ mm}$ ($\rho = 0.34$, $P = 0.17$) a
342 strong $[N]_{\text{DW}}$ and $[P]_{\text{ex}}$ relationship was evident for plots with $P_D > 100 \text{ mm}$ ($\rho = 0.66$, P
343 $= 0.002$: Figure 3d).

344 Irrespective of dry season precipitation amount, there was a strong relationship
345 between $[P]_{\text{DW}}$ and $[P]_{\text{ex}}$, but with the relationship tending to saturate at high $[P]_{\text{ex}}$ for
346 $P_D < 100 \text{ mm}$. In terms of foliar nitrogen:phosphorus ratios, for plots with $P_D < 100 \text{ mm}$

347 values were, on average, significantly higher than in $P_D > 100$ mm plots ($F_{(1,71)} = 17.41$,
348 $P < 0.0001$). There was also an inverse relationship between foliar nitrogen:phosphorus
349 ratio and $[P]_{ex}$ evident irrespective of dry season precipitation regime (Figures 3g, h).

350

351 $\delta^{15}N_F$ values of trees of the Fabaceae family

352 Of a total of 118 potentially N_2 -fixing Fabaceae examined, only 43 (or 36%) could be
353 inferred as actively fixing atmospheric nitrogen according to our criterion of $\delta^{15}N_F -$
354 $\delta^{15}N_F$ being 1‰ or greater (Table S3). Checking this against the percentage of non-
355 fixing Fabaceae that would incorrectly score as actively fixing according to this ‘1 per
356 mil criterion’ we found a false strike rate of about 10%, this suggesting that, if anything,
357 the real value was probably somewhat less than 36%.

358 Of the 48 plots with at least one putatively (nodulating) Fabaceae species having
359 been sampled, only 29 had at least one tree inferred to be actively fixing nitrogen (Table
360 S3). Most of these were characterised by young to intermediate age soils (21 out of 29
361 plots, Table S3), these often being Alisols with a low degree of weathering in
362 comparison to more mature soils, such as Ferralsols and Acrisols (Quesada et al. 2009;
363 2010). However, it is important to note that in five Ferralsol plots, evidence of some N_2
364 fixation was indicated (Table S3). Two of these plots (SIN-01, VCR-02) were located in
365 the State of Mato Grosso, two (TAP-123, TAP-04) in the State of Pará, and one (MAN-
366 12) in the Amazonas State, all inside Brazil (Table S1).

367 Applying Equation 1 to Fabaceae trees only, about half of the variance in the
368 observed $\delta^{15}N_F$ emerged was attributable to the plot in which the tree was growing, ca.
369 15% was due to genus and the remainder (35%) to the residual. With this restricted
370 dataset, variation at the species level could not be reasonably be included for analysis at

371 the lowest level of variation due to the generally low number of species sampled per
372 genus and/or trees sampled per species.

373 Along with the $\delta^{15}\text{N}_F$ values of the putatively (nodulating) Fabaceae species
374 ($\delta^{15}\text{N}_{\text{Fix}}$) and average $\delta^{15}\text{N}$ values of non-fixing species ($\delta^{15}\text{N}_{\text{nF}}$), we indicated the recent
375 N_2 -fixation status for the Fabaceae species using the $>1\text{‰}$ criterion as follow: F+,
376 legumes which were assumed to fix nitrogen during the survey; and F-, legumes whose
377 leaf $\delta^{15}\text{N}$ values indicated no nitrogen fixing during the survey (Table 1, Table S3).
378 Comparing foliar characteristics of the Fabaceae (nitrogen, phosphorus and their $\delta^{15}\text{N}_F$),
379 the only difference observed among F+, F- and $\delta^{15}\text{N}_{\text{nF}}$ was a significantly lower $\delta^{15}\text{N}_F$
380 ($F_{(2,71)} = 1.72, P = 0.19$) in F+ compared with both F- and $\delta^{15}\text{N}_{\text{nF}}$; the latter two not
381 differing from each other (Table 1). We also did not find any statistically significant
382 differences in $[\text{N}]_{\text{DW}}$ and $[\text{P}]_{\text{DW}}$ as well as in nitrogen:phosphorus ratios among them
383 (Table 1).

384

385 **Discussion**

386 *Overall trends in foliar $\delta^{15}\text{N}$*

387 Much of the variation in $\delta^{15}\text{N}_F$ (a range of 21‰) could be attributed to local conditions,
388 especially precipitation and soil characteristics, with little variation attributable to a
389 species' taxonomic identity/affiliation. The mean $\delta^{15}\text{N}_F$ (\pm standard deviation) of
390 $+3.1 \pm 2.3\text{‰}$ ($n = 1255$) found in our study for Amazon forest is significantly greater
391 than $\delta^{15}\text{N}_F$ typically reported for temperate forests. For example, in a survey involving
392 different temperate tree species of North America and Europe, $\delta^{15}\text{N}_F$ varied from -8‰
393 to $+4\text{‰}$ but usually less than 0‰ (Pardo et al. 2006). The mean difference in $\delta^{15}\text{N}_F$
394 between tropical and temperate forests was anticipated as it has already been shown that
395 N-rich/leaky forests have higher $\delta^{15}\text{N}_F$ than N-poor/tightly cycling forests due to losses

396 of ^{14}N in relation to ^{15}N in processes such as denitrification (Martinelli et al. 1999;
397 Houlton et al. 2006).

398

399 *Is the coupling of phosphorus and nitrogen cycles modulated by precipitation?*

400 Lowland tropical forest productivity is often considered to be more limited by
401 phosphorus than nitrogen (Vitousek 1984; Crews et al. 1995; Chadwick et al. 1999;
402 Hedin et al. 2003; McGroddy et al. 2004; Reich and Oleksyn 2004), especially in the
403 case of old and highly leached soils (Porder et al. 2007; Vitousek et al. 2010; Quesada
404 et al. 2012). Nevertheless, there are indications that productivity can also be limited by
405 nitrogen in some cases, especially in response to transient nitrogen limitation (Davidson
406 et al. 2007) and/or on younger soils where the phosphorus availability is relatively high
407 (Quesada et al. 2010; Mercado et al. 2011).

408 As pointed out earlier, Quesada et al. (2010) found that to ca. 30 mg kg⁻¹ of $[\text{P}]_{\text{ex}}$
409 there was a direct relation between this soil property and $\delta^{15}\text{N}_\text{S}$ (see Figure S1)
410 suggesting that at the lowest $[\text{P}]_{\text{ex}}$ there were potentially limitations on plant
411 productivity due to low levels of nitrogen availability which may have even exceeded
412 those expected as a consequence of low plant available phosphorus concentrations in the
413 soil. For these reasons, and considering that ca. 70% of $\delta^{15}\text{N}_\text{F}$ variance was attributed to
414 local conditions (soil and climate), and more than 60% of the foliar $\delta^{15}\text{N}_\text{F}$ variance was
415 explained by $\delta^{15}\text{N}_\text{S}$, and with a clear pattern between $[\text{P}]_{\text{ex}}$ and $\delta^{15}\text{N}_\text{S}$ previously found
416 by Quesada et al. (2010), it seemed useful to investigate the role of $[\text{P}]_{\text{ex}}$ in modulating
417 $\delta^{15}\text{N}_\text{F}$. For plots with $P_\text{D} < 100$ mm a similar trend exists between $\delta^{15}\text{N}_\text{P}$ and $[\text{P}]_{\text{ex}}$ as is
418 the case for $\delta^{15}\text{N}_\text{S}$ and $[\text{P}]_{\text{ex}}$. More specifically, in the interval from very low
419 concentrations up to 30-50 mg kg⁻¹ of $[\text{P}]_{\text{ex}}$, an increase in the $\delta^{15}\text{N}_\text{P}$ value was
420 observed, with the highest $\delta^{15}\text{N}_\text{P}$ values observed mainly in Ferralsols and Acrisols of

421 eastern Amazonia. Beyond this $[P]_{\text{ex}}$ threshold, similar to the $\delta^{15}\text{N}_\text{S}$, $\delta^{15}\text{N}_\text{P}$ then slightly
422 decreases, with this associated with progressively younger soils.

423 Plants on lowest $[P]_{\text{ex}}$ white-sandy soils in the Amazon region (sites with
424 relatively large annual precipitation) have already been noted to have low $\delta^{15}\text{N}_\text{F}$ (Pons et
425 al. 2007; Nardoto et al. 2008; Mardegan et al. 2009), perhaps due to a low availability
426 of nitrogen in these soils, as is also the case for other forests on Podzols, such as our
427 SCR-04 plot in Venezuela. Nitrogen limitations in such soils appears to be caused by
428 low rates of mineralisation and nitrification (Vitousek and Matson 1988; Luizão et al.
429 2004; Nardoto et al. 2008; Mardegan et al. 2009), in the presence of relatively high
430 levels of phosphorus availability in litter mats and humic horizons despite the low
431 overall $[P]_{\text{ex}}$ (Tiessen et al. 1994). In response to such conditions, both $[N]_{\text{DW}}$ and $[P]_{\text{DW}}$
432 were low (Figure 3c,d,e,f). Consistent with this are the results from a recent modelling
433 analysis using the RAINFOR data (Mercado et al. 2011). They found the only Podzol
434 site in their dataset (SCR-04) to be one of the few plots across the Amazon for which
435 photosynthetic productivity was limited by low foliar nitrogen rather than by
436 phosphorus, despite the vegetation at this site having low foliar phosphorus
437 concentrations (Table S1). This result was consistent with the suggestion of Quesada et
438 al. (2010) that, due to feedback effects of litter quality on nitrogen mineralisation rates,
439 the oldest and/or most chemically weathered forest soils of the Amazon may end up
440 supporting ecosystems with self-imposed limitations to productivity through low levels
441 of nitrogen availability.

442 As the $[P]_{\text{ex}}$ increased beyond its lowest observed values, $\delta^{15}\text{N}_\text{P}$ also increased,
443 reaching a maximum in the Ferralsols of Tapajós National Forest (TNF) and in
444 Caixuanã (TAP-123, TAP-04, CAX-06, Table S1). These three sites have an
445 intermediate $[N]_{\text{DW}}$ content, and a rather low $[P]_{\text{DW}}$ resulting in high foliar N:P ratios

446 (Table S1). This suggests that these forests are more limited by phosphorus than by
447 nitrogen (Silver et al. 2000), a result also consistent with the analysis of the dual
448 nitrogen-phosphorus photosynthesis limitation model of Mercado et al. (2011). Clay
449 soils in the TNF, such as Ferralsols, showed higher mineralisation, nitrification, and
450 denitrification than sandier soils of the same region (Silver et al. 2000) and Keller et al.
451 (2005) found the highest N₂O soil flux among tropical forests on these soils. Soil N₂O
452 fluxes have recently been linked to forest productivity and nitrogen availability (Wolf et
453 al. 2011). Taken together, this information suggests that nitrogen availability is
454 relatively high in these forests (Davidson et al. 2007). Given that high nitrogen
455 availability (Vitousek et al. 1989; Martinelli et al. 1999; Houlton et al. 2006; Pardo et al.
456 2006; Craine et al. 2009; Posada and Schuur 2011), and moderate soil water availability
457 constraints (Heaton 1987; Austin and Vitousek 1998; Handley et al. 1999; Amundson et
458 al. 2003; Nardoto et al. 2008; Craine et al. 2009; Posada and Schuur, 2011) are both
459 associated with higher $\delta^{15}\text{N}_F$, it therefore seems reasonable to suggest that the high
460 $\delta^{15}\text{N}_F$ and $\delta^{15}\text{N}_S$ values found on Ferralsols of the TNF are a consequence of relatively
461 clay-rich old soils combined with moderate soil water deficits and with phosphorus
462 limitation on plant productivity (Ometto et al. 2006; Nardoto et al. 2008; Quesada et al.
463 2012) leading to relatively high levels of both nitrogen availability and loss (Ometto et
464 al. 2006; Nardoto et al. 2008).

465 It is interesting to note that, as was observed for soils in Quesada et al. (2010),
466 beyond approximately 50 mg kg⁻¹ of [P]_{ex} there was a decline in the $\delta^{15}\text{N}_P$ in plots with
467 $P_D > 100$ mm. In this end part of the curve, trees were growing in plots where less
468 strongly weathered soils dominated (Umbrisols, Cambisols, Gleysols and Alisols,
469 Figure 3a). This is the same relationship as for nitrogen isotopes in the soil and, evoking
470 the same explanation presented by Quesada et al. (2010): that younger soils have not yet

471 experienced sufficient nitrogen losses from the system (Houlton et al. 2006) to enrich
472 such soils in ^{15}N atoms with a consequent increase in their $\delta^{15}\text{N}_\text{F}$ and $\delta^{15}\text{N}_\text{S}$.

473 Through analysing foliage of trees of the same plot we can, however, add another
474 layer of interpretation to that possible from soil analyses alone. Although $\delta^{15}\text{N}_\text{F}$ values
475 are clearly influenced by the $\delta^{15}\text{N}_\text{S}$, additional factors, such as soil nitrogen absorption
476 fractionation, internal plant processes (nitrogen retranslocation), and plant functional
477 differences might also influence patterns of nitrogen cycling (Robinson 2001). For
478 example, even though $[\text{N}]_{\text{DW}}$ were not greatly different when older soils were compared
479 with younger soils where $P_{\text{D}} < 100$ mm (Figure 3c), there was a general tendency for
480 $[\text{P}]_{\text{DW}}$ content to not positively correlate with $[\text{P}]_{\text{ex}}$, with $[\text{P}]_{\text{DW}}$ actually tending to
481 decrease only beyond a threshold of $[\text{P}]_{\text{ex}}$ ca. 200 mg kg^{-1} . Soils with highest $[\text{P}]_{\text{ex}}$ do
482 not necessarily support tropical forests with higher foliar phosphorus concentration than
483 those with lower levels of phosphorus availability - with dry-season precipitation being
484 a likely modulating factor. The very low foliar nitrogen:phosphorus ratios of the
485 vegetation on younger soils (Figure 3g) can, however, be taken as an indication of a
486 vegetation limited by nitrogen (Koerselman and Meuleman 1996; Gusewell 2004). The
487 stand-level photosynthesis modelling analysis of Mercado et al. (2011) also inferred that
488 some of the younger sites (Figure 3g) tended to be limited by nitrogen rather than
489 phosphorus, even though foliar nitrogen values were relatively high (Figure 3e,f). This
490 is consistent with the Walker and Syers's (1976) hypothesis, suggesting a stronger
491 nitrogen- than phosphorus-limitation in less developed soils, with their relatively low
492 $\delta^{15}\text{N}_\text{F}$ suggestive of a less leaky nitrogen cycle, but with phosphorus availability also
493 modulated by dry-season precipitation regime.

494 For plots with $P_{\text{D}} > 100$ mm there was a strong correlation between foliar $\delta^{15}\text{N}_\text{P}$
495 and $[\text{P}]_{\text{ex}}$ with nearly 0.7 of the variance in $\delta^{15}\text{N}_\text{P}$ explained by $[\text{P}]_{\text{ex}}$ (Figure 3b). In this

496 case, as was also observed for $P_D < 100$ mm, $\delta^{15}\text{N}_p$ were sometimes less than 1‰ at
497 very low $[\text{P}]_{\text{ex}}$. But contrary to $P_D < 100$ mm, the highest $\delta^{15}\text{N}_p$ values were found at the
498 highest $[\text{P}]_{\text{ex}}$ (Figure 3b) and with the highest $\delta^{15}\text{N}_p$ for the higher dry-season
499 precipitation plots ca. 2‰ less than the highest $\delta^{15}\text{N}_p$ observed for plots with $P_D < 100$
500 mm (Figures 3a,b). Thus, it seems that once differences in $[\text{P}]_{\text{ex}}$ are accounted for that
501 stand-level $\delta^{15}\text{N}_F$ tend to be reduced at higher P_D .

502 There have already been several studies similarly indicating that $\delta^{15}\text{N}_F$ values may
503 decrease with precipitation (Heaton 1987; Schulze et al. 1998; Austin and Vitousek
504 1998; Handley et al. 1999; Amundson et al. 2003; Santiago et al. 2004; Nardoto et al.
505 2008; Craine et al. 2009; Posada and Schuur 2011). Explanations for this effect are still
506 unclear, with explanations encompassing a range of factors including nutrient
507 availability, and leaf physiological traits (Santiago et al. 2004) and with Austin and
508 Vitousek (1998) hypothesising that in drier areas nitrogen cycling may be more open
509 compared to wetter areas; this leading to a $\delta^{15}\text{N}_S$ enrichment. Analysing seven lowland
510 neotropical forests plots along a gradient of P_A from ca. 2700 mm year⁻¹ to more than
511 9500 mm year⁻¹ Posada and Schuur (2011) suggested that at wetter sites there was
512 limitation by both nitrogen and phosphorus, which in turn caused a slow decomposition
513 rate due to poor litter quality, this then leading to carbon accumulation in the soil
514 (Vieira et al. 2011). As a result, nitrogen availability was argued to be lower at wetter
515 sites, leading to nitrogen-retentive forests, and lower $\delta^{15}\text{N}_S$ values. One possibility is
516 that this extra nitrogen is lost through complete denitrification of the nitrate pool
517 (Houlton et al. 2006).

518 Contrasting these results, Santiago et al. (2005) working along a precipitation
519 gradient from 1800 mm year⁻¹ to 3500 mm year⁻¹ in Panamá did not find any systematic
520 effects of rainfall on stand-level foliar $[\text{N}]_{\text{DW}}$, but with lower $\delta^{15}\text{N}_F$ occurring at the

521 higher precipitation sites (Santiago et al. 2004, 2005). This contradiction suggests that
522 there is a decoupling of the soil nitrogen cycle and leaf traits, suggesting, in turn, that
523 foliar nitrogen concentration and isotopic composition may depend more on leaf
524 structure and physiology than nitrogen availability (Santiago et al. 2005). Therefore, the
525 findings of Santiago et al. (2004, 2005) point to the fact that our interpretation should be
526 viewed with caution, although several other studies have shown a strong relation
527 between soil nitrogen availability and $\delta^{15}\text{N}_F$ (Vitousek et al. 1989; Martinelli et al. 1999;
528 Houlton et al. 2006; Pardo et al. 2006; Craine et al. 2009; Posada and Schuur 2011;
529 Wolf et al. 2011). Cernusak et al. (2009) confirmed by observation that for tropical trees
530 large differences in $\delta^{15}\text{N}_F - \delta^{15}\text{N}_S$ may occur as a consequence of considerable inter-
531 specific variation in the transpiration efficiency of nitrogen acquisition. This, therefore,
532 might explain, at some extent, the differences of rainfall effects in the relationship
533 between soil nitrogen and $\delta^{15}\text{N}_F$ in tropical forests.

534

535 *The role of Fabaceae in the tropical forests*

536 Because of their abundance, diversity, and N_2 -fixing ability the Fabaceae have often
537 been considered to play a prominent role in tropical forests (Vitousek et al. 2002; Hedin
538 et al. 2009; Barron et al. 2011). The monophyletic Fabaceae constitute one of the most
539 important botanical families in the Amazon region (Gentry 1988; ter Steege et al. 2006)
540 and the ability of some legumes to establish a symbiotic relationship with rhizobia make
541 this family even more important because of their ability to fix N_2 from the atmosphere
542 (Hoering and Ford 1960). Whether fixing or not, there are numerous reports of members
543 of this family typically having greater $[\text{N}]_{\text{DW}}$ than other trees (McKey 1994; Roggy,
544 Prevost, Garbaye, et al. 1999; Vitousek et al. 2002; Ometto et al. 2006; Nardoto et al.
545 2008; Fyllas et al. 2009) and it is thought that, because decomposition depends on the

546 litter quality (Santiago 2007; Cornwell et al. 2008), legume-rich forests should also
547 produce a nitrogen-rich litter, stimulating decomposition and liberating nitrogen for all
548 species present (Although we do note there may be exceptions to this general pattern;
549 e.g., Palm and Sanchez 1991).

550 Estimates of N₂-fixation in the Guiana lowland forests ranged from ca. 4 to 7 kg
551 ha⁻¹ year⁻¹ for some Amazonian Oxisols (Roggy, Prevost, Garbaye, et al. 1999; Pons et
552 al. 2007), which is equivalent to ca. 6% of the total annual nitrogen input to the forest.
553 On the other hand, several other studies have shown that putatively Fabaceae N₂-fixing
554 species often do not fix nitrogen from the atmosphere in mature, undisturbed tropical
555 forests (Sylvester-Bradley et al. 1980; Salati et al. 1982; Shearer and Kohl 1986;
556 Yoneyama et al. 1993; Högberg and Alexander 1995; Vitousek et al. 2002; Gehring et
557 al. 2005; Ometto et al. 2006; Nardoto et al. 2008; Barron et al. 2011). It has thus been
558 argued that it is only advantageous to Fabaceae to fix in these environments where the
559 cost of nitrogen fixation becomes affordable and/or the natural nitrogen cycle has
560 become disturbed (Sylvester-Bradley et al. 1982; Vitousek et al. 2002; Hedin et al.
561 2008; Barron et al. 2011).

562 Foliar $\delta^{15}\text{N}$ composition has been used for four decades as an indicator of nitrogen
563 fixation since the $\delta^{15}\text{N}$ of the air is approximately 0‰ and is generally lower than $\delta^{15}\text{N}_\text{S}$
564 (Hoering and Ford 1960; Amarger et al. 1977; Delwiche et al. 1979; Högberg 1986;
565 Shearer and Kohl 1986; Peoples and Craswell 1992; Sprent et al. 1996; Roggy, Prevost,
566 Goubiere, et al. 1999; Gehring et al. 2005; Pons et al. 2007; Unkovich et al. 2008). The
567 $\delta^{15}\text{N}_\text{F}$ of the fixing species is compared with the $\delta^{15}\text{N}_\text{F}$ of non-fixing species. If the
568 $\delta^{15}\text{N}_\text{F}$ of fixing species is significantly closer to 0‰ than the $\delta^{15}\text{N}_\text{F}$ of non-fixing species
569 this means that the fixing species is receiving some nitrogen from its symbiont
570 (Delwiche et al. 1979). This method is not, however, without its problems. First, it has

571 to be assumed that there is no fractionation from air-bacteria-plant. In most cases this
572 assumption appears to be true, or if not, the fractionation is rather small (Hoering and
573 Ford 1960; Högberg 1997). Second, as pointed out by Shearer and Kohl (1986) it has to
574 be assumed that the non-fixer species are taking up the same nitrogen form as the fixing
575 species (NH_4 , NO_3 , organic nitrogen forms), and that the $\delta^{15}\text{N}$ of the bulk soil is a good
576 indicator of the $\delta^{15}\text{N}$ of the inorganic or organic form that the plants are taking up. Both
577 assumptions are difficult to prove unequivocally, and most of the time, are probably, to
578 some degree, violated (Högberg 1997). Therefore estimates presented here are only a
579 rough indication of whether some nitrogen biological fixation occurred or not and need
580 to be interpreted with caution due to these methodological limitations (Table S3).

581 Nevertheless, our results based on $\delta^{15}\text{N}_F$ of potentially fixing Fabaceae and non-
582 fixing species confirm the low extent of N_2 -fixation in mature lowland tropical forests
583 across the Amazon Basin (Table S3), with the $\delta^{15}\text{N}_F$ analyses suggesting that only a
584 small portion of potentially N_2 -fixing Fabaceae fix N_2 . Where observed, this N_2 -
585 fixation was usually for trees growing on less weathered soils, which are characterised
586 by having a high $[\text{P}]_{\text{ex}}$, high total base reserves (Σ_{RB}) and often with some type of
587 physical constraint, which is expressed by the high value of the Π_1 index (see Quesada
588 et al. 2010 for details). These constraints include depth, structure, topography and
589 anoxia that are thought to translate into the field as characteristics that prevent plant
590 development such as a shallow soil and rooting depth, steep topography, and structural
591 and drainage deficiencies and it may be relevant that, in addition to there being a
592 tendency for a higher relative frequency of nodulation in the species in tropical
593 secondary forests (Gehring et al. 2005; Davidson et al. 2007; Barron et al. 2011) it also
594 seems that nodulation may be more common for undisturbed stands exposed to regular
595 flooding (Moreira et al. 1992; De Faria et al. 2010). Consistent with this observation,

596 Martinelli et al. (1992) and Kriebich et al. (2006) also found N₂-fixation to be important
597 in the Amazon várzea (flooded forests) and Koponen et al. (2003) found fixing
598 Fabaceae in a freshwater swamp forest in French Guiana. Likewise, Sprent (2001) has
599 emphasised the importance of flooding for N₂-fixation in legumes in the Orinoco Basin
600 and with James et al. (2001) describing flooding tolerant legumes from the Brazilian
601 Pantanal wetland, including one species that only nodulates under flooding conditions.
602 Taking a parallel with our younger soils mostly in the plots with $P_D > 100$ mm, we thus
603 suggest that soils with greater physical constraints that are – other characteristics being
604 similar – exposed to more frequent and longer periods of waterlogging associated with
605 anoxia, might explain the more frequent occurrence of actively fixing Fabaceae on such
606 soils. Here we note that pressurised or diffusive gas transport strategies are sometimes
607 capable of maintaining an adequate O₂ supply to the root system under anaerobic
608 conditions, and may also serve to maintain symbiotic N₂ fixation during these times
609 (Martinelli et al. 1992; James et al. 2001; Kriebich et al. 2006). Moreover, high rates of
610 denitrification under anaerobic conditions, occasional spatial N limitation and the
611 significant high seasonal N losses due to the exchange with rapid water flows all
612 support the notion that a seasonally waterlogged environment is one where a capability
613 for symbiotic N₂ fixation might be most beneficial (Roggy, Prevost, Garbaye et al.
614 1999; Vitousek et al. 2002).

615 We also speculate that the same may occur in the *baixio* of Manaus, an N-poor
616 system on sandy soils that is frequently inundated (Nardoto et al. 2008), even though
617 the relative abundance of putative N-fixers is not exceptionally high (Table S1).
618 However the negative $\delta^{15}\text{N}_F$ values of several plants in this area prevented the use of
619 $\delta^{15}\text{N}_F$ values as an indicator of N fixation by putatively N₂-fixing Fabaceae.

620 As discussed above, tropical forests on mature soils, such as the Ferralsols of
621 Brazil are generally considered phosphorus-poor and with nitrogen being relatively
622 abundant (Martinelli et al. 1999; Ometto et al. 2006; Nardoto et al. 2008; Quesada et al.
623 2010; Quesada et al. 2012) and it might therefore be expected that N₂-fixation rates
624 would be minimal (Vitousek et al. 2002; Houlton et al. 2008; Hedin et al. 2009; Baron
625 et al. 2011). Our data tend to confirm this view with N₂-fixing Fabaceae being more
626 common on less developed soils, but nevertheless, it is clear that some fixation may
627 occur in old mature tropical soils in some circumstances. The Ferralsols for which we
628 found some indications of N₂-fixation occurring also tended to have slightly higher
629 relative abundances of putative N₂-fixing Fabaceae than the Ferralsols on which we
630 failed to detect any N₂-fixation. One possible explanation for this apparent anomaly is
631 that on the oldest most strongly weathered Ferralsols, a gradual transition from
632 phosphorus to nitrogen limitation has already occurred, this being due to an
633 immobilisation of nitrogen associated with high lignin/tannin contents of decomposing
634 foliage growing on the oldest soils (Quesada et al. 2010).

635 As we measured the relative abundance of potentially N₂-fixing Fabaceae in most
636 of our plots (Table S1) we can also use our data here to provide a ‘back of the envelope’
637 estimate of the extent of N₂-fixation by Amazon forest. Noting that, on average, only
638 8% of the tree population consists of putative N₂-fixers (Table S1) and with only 36%
639 of such trees fixing nitrogen and with the differences in $\delta^{15}\text{N}_F$ in Table S3 suggesting
640 that at most those trees fixing nitrogen obtain 30% of their total N from the atmosphere,
641 then the total amount of N₂ fixed should be ca. $0.08 \times 0.35 \times 0.03$, or 1% of the average
642 nitrogen utilised by the Amazon forest each year. From recent work of Aragão et al.
643 (2009) estimated that, although somewhat variable, leaf and fine-root production
644 account for about 70% of total net plant primary production, a reasonable overall value,

645 being about $10 \text{ Mg C ha}^{-1} \text{ year}^{-1}$ which for a plant carbon concentration of 50% with
646 $[\text{N}]_{\text{DW}} = 20 \text{ mg g}^{-1}$ (Fyllas et al. 2009) and assuming a similar nitrogen concentration for
647 fine roots as foliage (Cuevas and Medina 1988) yields an estimate for a (leaf + fine root)
648 nitrogen requirement of $0.01 \times 2 \times 10 \times 20 \times 0.7$, or $2.8 \text{ kg N ha}^{-1} \text{ year}^{-1}$. To it must be
649 added the nitrogen requirements for new wood growth. Measurements of nitrogen of
650 tropical tree stem are very scarce, but taking a value of 1 mg g^{-1} based on what little
651 data are available - mostly from Australian tropical/subtropical forests (Meerts 2002) -
652 then calculating using the nitrogen requirement for new wood production of stems,
653 branches and coarse roots as for leaves and fine roots above, we obtain a value of
654 around $0.1 \text{ kg N ha}^{-1} \text{ year}^{-1}$. Taking every assumption made above to account for the
655 potential input of nitrogen via symbiotic biological fixation, we therefore estimate the
656 potential rate of N_2 -fixation by the Fabaceae Amazon trees of being ca. $3 \text{ kg N ha}^{-1} \text{ year}^{-1}$
657 ¹. This is likely an overestimate as retranslocation of nitrogen from senescing tissues has
658 not been considered, but is still less than the 4 to $7 \text{ kg N ha}^{-1} \text{ year}^{-1}$ for lowland tropical
659 forests of Guiana and Rondônia (Roggy, Prevost, Garbaye, et al. 1999; Cleveland et al.
660 2010), and much less than the estimate of Cleveland et al. (1999) for tropical forest
661 symbiotic nitrogen fixation of $16 \text{ kg N ha}^{-1} \text{ year}^{-1}$. It is, however, important to realise
662 that the global average value for tropical forest symbiotic N_2 -fixation presented in
663 Cleveland et al. (1999) was an indirect estimate; having being extrapolated from
664 measurements of free-living N_2 -fixation for a solitary Amazon forest site (Jordan et al.
665 1980).

666 Our estimate here also differs vastly from those predicted from a self-proclaimed
667 ‘unifying framework’ for N_2 fixation where values for lowland tropical forest of 20 to
668 $25 \text{ kg N ha}^{-1} \text{ year}^{-1}$ are suggested (Houlton et al. 2008). That model is based on the
669 simple assumption that N_2 -fixing legumes maintain their advantage over other tropical

670 tree species by virtue of being able to have higher rates of release of phosphatase
671 enzymes enabling access to phosphorus pools that non N₂-fixers do not. There are,
672 however, numerous other means by which plants can improve their access to
673 phosphorus, for example through organic acid exudation and/or mycorrhizal
674 associations (Lloyd et al. 2001; Turner 2008). There may also be other characteristics of
675 members of the Fabaceae contributing to their success in tropical forest systems as is
676 also suggested by the relatively high abundance of non-nodulating Fabaceae in the
677 Amazon (Table S1). For example, two of the fastest-growing of all tree genera in
678 Amazonia – *Inga* and *Tachigali* – are often characterised by the presence of a symbiotic
679 relationship with ants who aggressively defend their leaves against insect and other
680 herbivores (Gentry 1993), and the most abundant genus, *Inga*, also being characterised
681 by a relatively rare but highly efficient photosynthetic xanthophyll cycle (García-
682 Plazaola et al. 2007). Moreover, many members of the Fabaceae have compound leaves
683 of potential benefit under nutrient poor or water-stressed conditions (Malhado et al.
684 2010), also often being with large seeds conferring advantages on nutrient poor and/or
685 sandy soils (ter Steege et al. 2006). In this respect it is interesting to note that Corby et
686 al. (2011) reported that seed weight was higher and the concentration of nitrogen in the
687 seed was lower in non-nodulating Fabaceae compared to those that cannot nodulate but
688 that the seeds of non-nodulating species had a higher absolute nitrogen content than
689 those of nodulating species.

690 Finally, we note that our results here regarding the importance of N₂-fixing
691 Fabaceae in influencing the nitrogen cycle of the Amazon forest probably do not apply
692 to the tropical forests of other regions of the world. For example, Raven and Pothill
693 (1981) have argued that as the rainforest flora of parts of Asia was well established
694 before legumes were able to reach the area, then there was no great pressure for nitrogen

695 fixation in this region. Thus, relatively few N₂-fixing legumes have penetrated these
696 habitats. This also seems to be the case for many parts of Africa where non-nodulating
697 legumes are often more abundant in tropical forests than putative N₂-fixing Fabaceae
698 (Sprent 2009); and with ectomychorrhizae substituting as the principle mode of
699 symbiotic nitrogen acquisition (Sprent 2007). By contrast, around the time that
700 symbiotic N₂-fixation is thought to have evolved in the Fabaceae ca. 55 million years
701 ago (Ma), not only would the global climate have been extremely favourable for plant-
702 microbe carbon/nitrogen trading with high temperatures and high [CO₂] also prevailing
703 (Sprent 2007) but, especially when considered in conjunction with the wetter climate
704 (Bowen et al. 2004) the transformation in Amazonia from an ancient 'cratonic' to a
705 much more dynamic 'Andean' dominated landscape (Hoorn et al. 2010) around the
706 same time also providing uniquely favourable conditions for the evolution of legume
707 symbiosis in the neotropics for at least 30 Ma afterwards: These conditions being the
708 continual emergence of nitrogen-depauperate, disturbed and often waterlogged
709 landscapes, especially in western Amazonia as a consequence of proto-Andean uplift
710 and the associated geomorphological instability. As would be suggested by the
711 hypothesis of Walker and Syers (1976), under such circumstances the development of
712 an ability to acquire atmospheric N₂ and hence overcome potential ecosystem level
713 nitrogen limitations in the young forests emerging in Western Amazonia may have
714 provided an unprecedented opportunity for N₂-fixing Fabaceae to evolve and proliferate
715 in an otherwise resource-rich environment. With many of these legume lineages then
716 having survived to the present through a range of adaptations to the continually
717 changing Amazon tropical forest environment as outlined above, the clade has been able
718 to radiate and persist as a dominant taxonomic group. We suggest that, because of this
719 presence of N₂-fixing Fabaceae in the Amazon Basin over the last 50 Ma, that nitrogen

720 has now accumulated in many of the soils of the Amazon Basin to the extent that N₂
721 fixation is now rarely actually required. This idea is consistent with the current
722 distribution of Fabaceae that is considered to relate more to factors, such as rainfall and
723 temperature, than to rafting of land masses; it is also in accordance with the tendency of
724 Fabaceae establishing in seasonally dry areas prior to their colonisation in the moist
725 tropical forests (Schrire, Lavin, Lewis 2005; Schrire, Lewis, Lavin 2005).

726

727 *Conclusions*

728 The high number of plots encompassing different soil types and precipitation regimes
729 coupled with the high number of plants analysed in this study, has allowed us to
730 untangle the complex factors influencing $\delta^{15}\text{N}_F$ values in one of the most important
731 tropical forest regions of the world.

732 It had been known from several previous studies that mean annual precipitation
733 had an important influence on the $\delta^{15}\text{N}_F$ values. However, our study advanced the
734 understanding of the role of climate factors by showing that for tropical forests
735 precipitation during the dry season has a strong influence on the relationship between
736 $\delta^{15}\text{N}_F$, $[\text{N}]_{\text{DW}}$, $[\text{P}]_{\text{DW}}$ and $[\text{P}]_{\text{ex}}$. We have also shown for the first time that, through
737 effects on $[\text{P}]_{\text{ex}}$, soil age has a fundamental effect in influencing not only foliar nitrogen
738 and phosphorus concentrations (Fyllas et al. 2009), but also foliar $^{15}\text{N}:^{14}\text{N}$ ratios.

739 Finally, our study has confirmed the low incidence of N₂ fixation by Fabaceae
740 members, irrespective of whether they have this ability or not. We also show that
741 although young less-weathered soils have a high foliar phosphorus and nitrogen content,
742 they have a low nitrogen:phosphorus ratio, potentially making them more limited by
743 nitrogen than by phosphorus, with, generally speaking, more nitrogen fixation in these
744 younger soils than in old weathered soils that dominate the tropics. Overall, we estimate

745 an average maximum symbiotic N₂-fixation rate occurring within Amazon forest at
746 maximum 3 kg N ha⁻¹ year⁻¹. This is necessarily a rough estimate is much less than that
747 suggested by several other scaling and/or modelling analyses which may have been
748 based on unrealistic assumptions. Due to the unique tectonic history of the Amazon
749 Basin our results cannot be simply extrapolated to the tropical forest of other continents
750 with rates of symbiotic nitrogen fixation by tropical forests on other continents probably
751 even less than those calculated here.

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1257 Table Legends

1258

1259 Table 1. Average foliar traits of Fabaceae trees grouped according the capability of

1260 symbiotic nitrogen fixation and current status of fixation. See Table S3 for details.

1261 Different superscript letters in the same column indicate significance difference at 5%

1262 level by using the Tukey's HSD (Honestly significant difference) test for unequal

1263 variance.

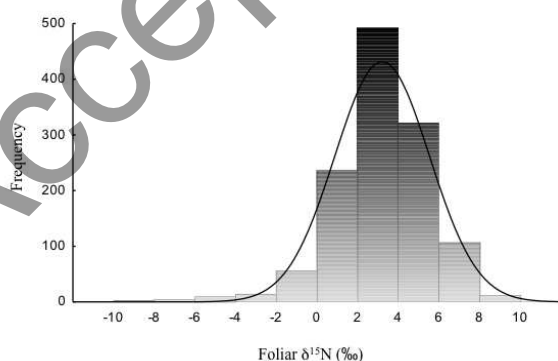
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1265 Figure Legends

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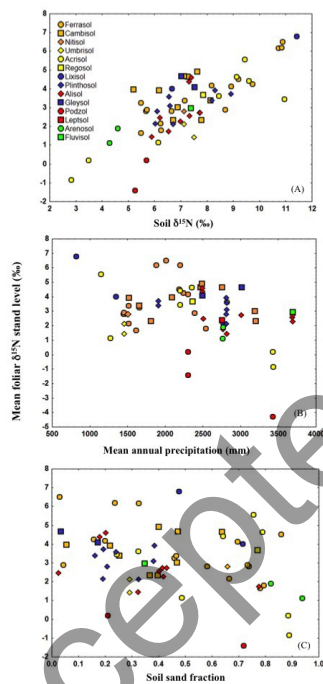
1267 **Figure 1.** Frequency distribution of foliar nitrogen natural isotopic abundance ($\delta^{15}\text{N}_F$)

1268 for 1255 trees sampled across Amazonia.



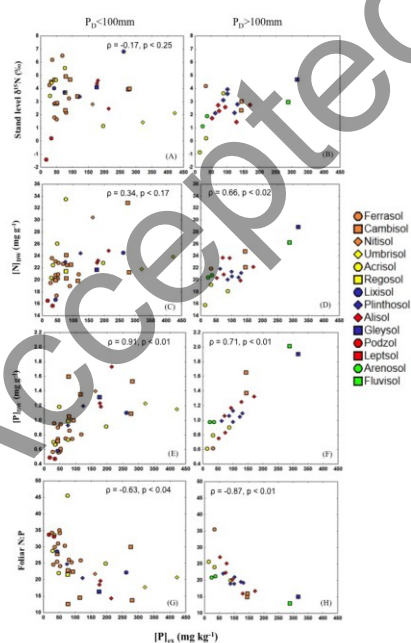
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1270 **Figure 2.** Scatter-plot of average $\delta^{15}\text{N}_F$ in the same plot ($\delta^{15}\text{N}_P$) versus (A) soil nitrogen
1271 natural isotopic abundance ($\delta^{15}\text{N}_S$); (B) mean annual precipitation (P_A); and (C) soil
1272 sand fraction in the sampled plots in Amazonia. Circles represent old weathered soils
1273 (total sum of bases 0 to 100 $\text{mmol}_c \text{kg}^{-1}$ clay, and total P < 200 mg kg^{-1}); diamonds
1274 represent intermediate weathered soils (total sum of bases 100 to 400 $\text{mmol}_c \text{kg}^{-1}$ clay,
1275 and total P < 600 mg kg^{-1}); and squares represent young less weathered soils (total sum
1276 of bases > 400 $\text{mmol}_c \text{kg}^{-1}$ clay, and total P > 600 mg kg^{-1}). Soil data from Quesada et al.
1277 (2010).
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1286 **Figure 3.** Scatter-plots of soil extractable phosphorus $[P]_{\text{ex}}$ versus foliar traits for plots
 1287 with a mean monthly precipitation during the driest quarter (P_D) of < 100 mm ($P_D < 100$
 1288 mm) and for $P_D > 100$ mm: (A) and (B) soil $[P]_{\text{ex}}$ versus soil nitrogen natural isotopic
 1289 abundance ($\delta^{15}\text{N}_s$); (C) and (D) soil $[P]_{\text{ex}}$ versus foliar nitrogen concentrations ($[\text{N}]_{\text{DW}}$);
 1290 (E) and (F) soil $[P]_{\text{ex}}$ versus foliar phosphorus concentrations ($[\text{P}]_{\text{DW}}$); and (G) and (H)
 1291 soil $[P]_{\text{ex}}$ versus foliar nitrogen:phosphorus ratios (foliar N:P). Data for soil $[P]_{\text{ex}}$ from
 1292 Quesada et al. (2010); foliar nitrogen, phosphorus, and nitrogen:phosphorus from Fyllas
 1293 et al. (2009). Circles represent older, more weathered soils (total sum of bases 0 to 100
 1294 $\text{mmol}_c \text{kg}^{-1}$ clay, and total phosphorus $< 200 \text{mg kg}^{-1}$); diamonds represent intermediate
 1295 weathered soils (total sum of bases 100 to 400 $\text{mmol}_c \text{kg}^{-1}$ clay, and total phosphorus $<$
 1296 600mg kg^{-1}); and squares represent young less weathered soils (total sum of bases $>$
 1297 $400 \text{mmol}_c \text{kg}^{-1}$ clay, and total phosphorus $> 600 \text{mg kg}^{-1}$). Soil chemistry data from
 1298 Quesada et al. (2010).
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1303 Table 1. Average foliar traits of Fabaceae trees grouped according the capability of
 1304 symbiotic nitrogen fixation and current status of fixation. See Table S3 for details.
 1305 Different superscript letters in the same column indicate significance difference at 5%
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 1307 variance.

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Potential status	Confirmed status	N^b	$\delta^{15}N_F$ (%)	$[N]_{DW}$ (mg kg ⁻¹)	N:P ^d	$[P]_{DW}$ (mg g ⁻¹)
Fixer	F+	14	+1.4 ^b	26.4 ^a	26 ^a	1.1 ^a
Fixer	F-	34	+3.5 ^{ab}	25.3 ^a	27 ^a	1.0 ^a
Non-fixer	nF	28	+2.9 ^{ab}	21.9 ^a	23 ^a	1.2 ^a

1311 Fixer, putatively (nodulating) Fabaceae species; non-fixer, non-fixing Fabaceae species;
 1312 F+, legumes which were assumed to fix nitrogen during the survey (>1‰ criterion); F-,
 1313 legumes whose leaf $\delta^{15}N$ values indicated no nitrogen fixing during the survey; nF, non-
 1314 fixer; N , number of samples; N:P, foliar nitrogen:phosphorus ratio; different letters in
 1315 superscript indicate significant differences in a same column.

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