Divergent patterns of foliar δ^{13}C and δ^{15}N in *Quercus aquifolioides* with an altitudinal transect on the Tibetan Plateau: an integrated study based on multiple key leaf functional traits

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Abstract

Aims

With a close association with plant water availability, foliar δ^{13}C had been investigated extensively in alpine regions; however, foliar δ^{15}N has rarely been concurrently used as an indicator of plant nitrogen availability. Due to the positive correlations between leaf nitrogen content and foliar δ^{13}C and δ^{15}N found in previous studies, we expected that they should show consistent patterns along an altitudinal gradient.

Methods

To test our hypothesis, we measured foliar δ^{13}C and δ^{15}N in conjunction with multiple key leaf functional traits of *Quercus aquifolioides*, a dominant species of alpine forest on the eastern slopes of the Sygera Mountains, southeastern Tibetan Plateau from 2500 to 3800 m.

Important findings

(i) Contrary to our hypothesis, foliar δ^{13}C exhibited a significant positive linear relationship with altitude; in contrast, foliar δ^{15}N initially increased and subsequently decreased with altitude, the change in trend occurring around 3300 m. (ii) Our analyses indicated that leaf internal resistance and stomatal conductance, rather than photosynthetic capacity indicated by leaf N concentration, apparently explained the altitudinal variation in foliar δ^{13}C, while differences in foliar δ^{15}N were likely the result of soil N availability. (iii) Principal component analysis revealed a clear association between δ^{13}C and a tradeoff between water loss and carbon gain, indicated by traits related to gas exchange such as leaf thickness, density, stomatal properties. In contrast, the second axis was associated with δ^{15}N and nitrogen acquisition strategy in *Q. aquifolioides* across its altitudinal distribution, represented by traits related to nitrogen concentration and stomata per gram of leaf nitrogen.

Keywords: elevation, carbon and nitrogen isotopes, leaf internal resistance, leaf mass per unit area (LMA), stomatal conductance, trade-off

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INTRODUCTION

The relative proportions of stable isotopes of carbon and nitrogen can provide insights into nutrient and moisture dynamics in terrestrial ecosystems, because both isotopes are involved in several fundamental biogeochemical processes and are sensitive to environmental conditions (Handley *et al.* 1999). Therefore, variations in these

isotopes have been used in a number of studies to explore plant responses to changing climates and environmental gradients (Adams and Grierson 2001; Dawson *et al.* 2002). In particular, it has been suggested that they vary along altitudinal gradients in alpine regions (Cordell *et al.* 1998; Cordell *et al.* 1999; Hultine and Marshall 2000; Körner *et al.* 1988).

As an important indicator of plant long-term water use efficiency, foliar $\delta^{13}C$ at intra-specific level showed a strong and consistent increase with altitude (Hultine and Marshall 2000; Körner *et al.* 1988, 1991; Marshall and Zhang 1994; Sparks and Ehleringer 1997; Vitousek *et al.* 1990; Warren *et al.* 2001), although a nonlinear relationship has also been found in Asian alpine tree species (Li *et al.* 2009; Qiang *et al.* 2003; Zhao *et al.* 2008). Meanwhile, variation in key leaf functional traits, such as leaf mass per unit area (LMA), leaf nitrogen concentration per unit mass or area (N_{mass}, N_{area}), stomatal density (SD) that control to resource acquisition (i.e. carbon, nitrogen and water) and represent relatively independent aspects of plant ecological 'strategy' (Wright *et al.* 2004), has also been widely examined within alpine tree species, and these functional traits showed strong correlations with foliar $\delta^{13}C$. For instance, LMA and N_{area} generally increase with altitude, and positively correlate with foliar $\delta^{13}C$; in contrast, SD declines with altitude in many broad-leaved and coniferous woody species, and shows a negative relationship with foliar $\delta^{13}C$ (Cordell *et al.* 1998; Hultine and Marshall 2000; Körner 1989; Read *et al.* 2014; Vitousek *et al.* 1990). It has been suggested that physiological mechanisms responsible for carbon isotopic variation are associated with shifts between supply and demand for carbon dioxide within the leaves (Hultine and Marshall 2000). Low SD and high LMA reduce leaf stomatal conductance and increase the distance of CO_2 transport to sites of carboxylation, respectively, and thus reduce CO_2 supply to the chloroplasts and subsequently increase $\delta^{13}C$ of fixed carbon in leaves (Hultine and Marshall 2000; Vitousek *et al.* 1990). On the other hand, leaves with high leaf N_{mass} and/or N_{area} tend to have higher photosynthetic capacity because the protein in the photosynthetic apparatus represents up to 75% of total leaf organic nitrogen (Evans 1989; Poorter and Evans 1998), and thus have higher demand for CO_2 and subsequently have higher $\delta^{13}C$. To our knowledge, there have been no other investigations into the relative contribution of these different mechanisms to carbon isotope discrimination along altitudinal gradients in individual species.

In contrast to $\delta^{13}C$, $\delta^{15}N$ is associated with terrestrial N cycling (Robinson 2001) and has been shown, in numerous studies, to vary along environmental gradients at regional to global scales (Aranibar *et al.* 2004; Cheng *et al.* 2009; Craine *et al.* 2009; Handley *et al.* 1999; Houlton *et al.* 2006; Murphy and Bowman 2009; Swap *et al.* 2004; Yang *et al.* 2013). For instance, because warm, dry ecosystems might have a relatively open nitrogen cycle and thus higher soil N availability

(Robinson 2001), foliar $\delta^{15}N$ increases with decreasing mean annual precipitation and with increasing mean annual temperature (MAT) across MAT $\geq -0.5°C$ (Craine *et al.* 2009; Handley *et al.* 1999). Surprisingly, there have been very few studies of plant $\delta^{15}N$ at intraspecific level along altitudinal gradients. Sah *et al.* (2003) found that $\delta^{15}N$ in the needles of *Pinus roxburghii* declined along an altitudinal gradient from 1200 to 2200 m in the Kathmandu valley of Nepal. In contrast, Vitousek *et al.* (1989) found no differences in $\delta^{15}N$ in *Metrosiderous* collected from different altitudes along the Hawaiian coast. Although there is much published data about nitrogen isotopes in plants, at both regional and global scales, the effects of environmental variables on N isotope ratios are still far from complete. In general, it has been suggested that leaf $\delta^{15}N$ is positively correlated with leaf N concentration and soil N availability at various spatial scales: across 15 temperate and tropical forest sites (Martinelli *et al.* 1999); within and among plant species at Glacier Bay, Alaska (Hobbie *et al.* 2000); across 67 grasslands in four regions of the world (Craine *et al.* 2005); within and among species and temperate forest stands (Pardo *et al.* 2006); among herbaceous species in temperate grassland (Craine *et al.* 2012). Specifically, in a research review, Craine *et al.* (2009) found that $\delta^{15}N$ increased with increasing foliar N concentration at the global scale. However, so far, few studies have considered both leaf $\delta^{13}C$ and $\delta^{15}N$ simultaneously in alpine regions, and clearly, such an integrated study of both $\delta^{15}N$ and $\delta^{13}C$, as well as other key leaf functional traits, would contribute to our understanding of plant ecological strategies, such as carbon, water and nitrogen use. *Quercus aquifolioides* Rehder & E.H. Wilson is a dominant evergreen sclerophyllous species of alpine forests in the Tibetan Plateau and adjacent Hengduan Mountains, growing across a wide range of altitudes, from around 2000 to 4500 m (Zhou *et al.* 1995). Therefore, *Q. aquifolioides* is an excellent species in the alpine region for us to examine the intraspecific variation in foliar $\delta^{13}C$, $\delta^{15}N$ and other key leaf functional traits across altitudes. Due to the positive relationships between leaf N content and leaf $\delta^{15}N$ and $\delta^{13}C$, as shown above, we hypothesized that they should increase consistently along such an altitudinal gradient.

In this study, in order to test our hypothesis that foliar $\delta^{13}C$ and $\delta^{15}N$ could exhibit consistent patterns across an altitudinal gradient, we therefore measured foliar $\delta^{13}C$, $\delta^{15}N$ and other foliar traits in *Q. aquifolioides* at altitudes ranging from 2500 to 3800 m. Firstly, we examined whether $\delta^{13}C$, $\delta^{15}N$ and other leaf functional traits exhibited similar trends in response to increasing altitude. Then, we addressed two issues: (i) identifying key functional traits that caused altitudinal variation in $\delta^{13}C$ and $\delta^{15}N$; and (ii) determining the ecological consequences (e.g. water and nitrogen use strategies) of the differences in foliar $\delta^{13}C$ and $\delta^{15}N$, along with other leaf functional traits, across the altitudinal gradient.

MATERIALS AND METHODS

Field sites

In September 2007, leaves of *Q. aquifolioides* were sampled from 13 sites between 2500 and 3800 m, covering a wider altitudinal range than in previous studies of oak species (Li *et al.* 2009; Zhang *et al.* 2005), on the eastern slopes of the Sygera Mountains, southeastern Tibetan Plateau. This represented the entire altitudinal distribution range of *Q. aquifolioides* in this region. In order to avoid topological effects, all our samples were collected from the sunny eastern slopes. Climate data at three elevations, representing the low (3000 m), middle (3500 m) and high (3800 m) altitudes, were collected from local meteorological stations located within the Sygera region. The MAT for the low, middle and high altitudes was 8.6, 1.8 and −0.73°C, respectively. The corresponding mean annual precipitation (MAP) values were 660, 800 and 1134 mm. These values indicate that MAT declines linearly and MAP increases with increasing altitude in this region.

Mature leaves were collected from the south side of the crowns using a pruning pole. The slope of the land at the sites was 30–40%, with samples collected from 13 sites at nearly every 100 m from 2500 to 3800 m. At each site, 100 leaves from each of 5–10 individual mature trees were selected from the upper third of the canopy.

Leaf morphology

At each site, the leaf area (LA), mass (dry weight, LM) and leaf thickness (LT) of 10 leaves from each tree were determined. LA was measured using an LI-3000A portable area meter (Li-Cor, Lincoln, NE) and LT was measured with a digital caliper in the field. The measured samples were then placed in plastic bags and taken to the laboratory. Leaf samples were oven-dried for 48 h at 80°C until they reached constant weight, and then LM was determined and LMA (the ratio of dry weight to LA) was calculated.

Stable carbon and nitrogen isotope compositions

For stable carbon and nitrogen isotope analysis, dried leaf samples were ground to a fine powder with a Tissuelyzer (Retsch, Haan, Germany) and each sample was split into two subsamples, one of which was sent to the Key Laboratory of Western China's Environmental Systems (Ministry of Education), Lanzhou University, for the determination of δ¹³C and δ¹⁵N. All analyses were carried out with an elemental analyzer EA1108 (Carlo Erba, Milano, Italy) attached to a Delta Plus C isotope mass spectrometer and using a CONFELO Ⅱ interface (Thermo Finnigan MAT GmbH, Bremen, Germany). Carbon and nitrogen isotope ratios were reported as deviations from the standard Pee_Dee Belemnite (PDB) and atmospheric N₂ (defined as being 0‰), respectively, and expressed as per mil (‰). Therefore, the isotopic composition of a sample (δ¹³C or δ¹⁵N) was calculated as the ratio (‰):

$$\delta^{13}C_p(\delta^{15}N_p) = [(R_{sample} / R_{standard}) - 1] \times 1000$$

where R_{sample} and $R_{standard}$ are the ¹³C:¹²C (¹⁵N: ¹⁴N) ratios of the sample and the PDB (atmospheric N₂) standard, respectively.

Leaf carbon and nitrogen concentrations

Carbon and nitrogen concentrations of the leaves were determined, using the second set of leaf subsamples, by the Analytical Testing Center, Lanzhou University, China, with a CHN analyzer (Vario EL, Elementar, Germany). Leaf C and N concentrations per unit mass (C_mass, N_mass) were calculated, and then leaf C and N concentrations per unit area (C_area, N_area) were also calculated by multiplying C_mass and N_mass, respectively, by the LMA.

Stomatal measurements

Ten leaves preserved in FAA solution (38% Formaldehyde 5 ml: Glacial acetic acid 5 ml: 70% Alcohol 90 ml) per individual were first bleached with a 4% sodium hypochlorite solution, and then immersed in tap water to remove the mesophyll and epicuticle, because leaves of *Q. aquifolioides* are hypostomatous. The remaining cuticle was then stained with safranin and mounted in glycerin on a microscope slide. Screening was then conducted using a BX51 light microscope (Olympus, Shinjuku-ku, Tokyo, Japan) equipped with a digital camera DP71, at magnifications of ×10 and ×40. The stomatal density (SD, number of stomata mm⁻²) and the length of stomata (SL, guard cell length, μm) were measured on micrographs using the Image-Pro Plus 5.0 software. We also calculated stomatal number per gram of nitrogen (St/N) and per unit leaf mass (St/LM) by dividing SD by N_area and LMA (Hultine and Marshall 2000), respectively.

Statistical analyses

We performed two sets of analyses. First, we characterized the relationships between elevation and the measured variables, and between foliar δ¹³C, δ¹⁵N and other traits using linear univariate regressions of the mean population values. We then used the principal component analysis (PCA) to assess the ecological strategy associated with the altitudinal variation in leaf traits within *Q. aquifolioides*. All statistical analyses were conducted in SPSS 16.0 (SPSS lnc., Chicago, IL).

RESULTS

Altitudinal variation in foliar δ¹³C and δ¹⁵N in association with other leaf traits

As altitude increased, foliar δ¹³C appeared to increase (Fig. 1A), while stomatal parameters including SD, SL and St/LM, all declined markedly (Fig. 1C–F). Leaf morphological traits such as LMA, LM and LT increased significantly with increasing altitude (Fig. 1B, 2B and C); whereas LA exhibited the opposite trend (Fig. 2A). In addition, C_area increased significantly with increasing altitude (Fig. 2D), whereas C_mass exhibited no such trend ($R^2 = 0.177$, $P = 0.152$).

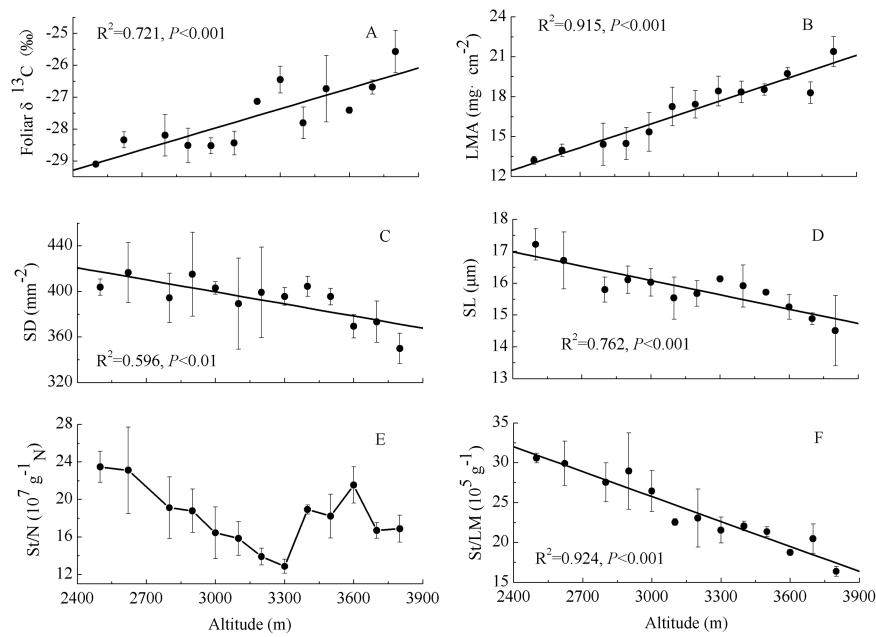**Figure 1:** relationships between altitude and (**A**) foliar $\delta^{13}C$, (**B**) leaf mass per unit area (LMA), (**C**) stomatal density (SD), (**D**) the length of stomata (SL), (**E**) number of stomata per gram of leaf nitrogen (St/N) and (**F**) number of stomata per leaf mass (St/LM) in *Quercus aquifolioides*. Values are means ± standard deviation.
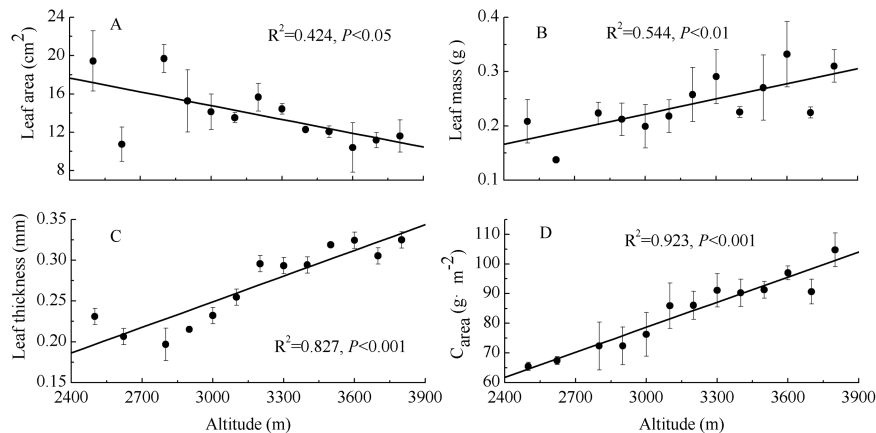

Figure 2: relationships between altitude and (**A**) leaf area, (**B**) leaf mass, (**C**) leaf thickness and (**D**) leaf C concentration per unit area (C_{area}) in *Quercus aquifolioides*. Values are means ± standard deviation.

By contrast, the relationships between foliar $\delta^{15}N$, N_{mass}, N_{area}, C:N, St/N and altitude were nonlinear, with a clear transitional zone at around 3300 m (Fig. 3, 1E; Table 1). Values of $\delta^{15}N$, N_{mass} and N_{area} increased linearly with altitude at low altitudes (up to 3300 m) and declined linearly, although not significantly, at high altitudes (above 3300 m). Conversely, C:N and St/N initially declined and then increased with altitude.

Relationships between foliar $\delta^{13}C$, $\delta^{15}N$ and other traits

Foliar $\delta^{13}C$ was significantly correlated with several leaf traits, including the morphological variables and stomatal

parameters which affect the internal resistance and stomatal conductance of leaves (Table 2). For example, foliar $\delta^{13}C$ exhibited significant positive correlations with LM, LT, LMA, and C_{area} and negative relationships with SD, SL and St/LM; in contrast, it exhibited relatively weak correlations with C_{mass} and LA ($P = 0.053$ and $P = 0.094$, respectively), but no correlations with N_{mass}, N_{area}, C:N and St/N (Data not shown). This suggests that altitudinal variations in $\delta^{13}C$ could be caused by leaf internal resistance and stomatal conductance, rather than by photosynthetic capacity indicated by leaf N.

Foliar $\delta^{15}N$ was also correlated with leaf traits, in particular N_{mass}, N_{area}, C:N and St/N (Table 3). It exhibited strikingly positive relationships with N_{mass} and N_{area}, and negative
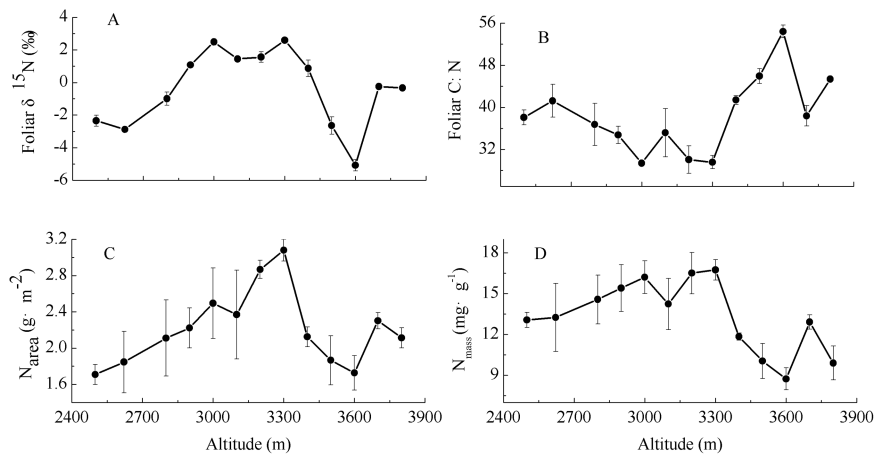
Figure 3: relationships between altitude and (**A**) foliar $\delta^{15}N$, (**B**) foliar C:N, (**C**) leaf N concentration per unit area (N_{area}) and (**D**) leaf N concentration per unit mass (N_{mass}) in *Quercus aquifolioides*. Values are means ± standard deviation.

Table 1: regression equations of physiological leaf traits across the two altitudinal ranges (low altitudes: below 3300 m; and high altitudes: above 3300 m) for alpine shrub species (*Quercus aquifolioides*).

Altitude	Statistic	$\delta^{15}N$	N_{mass}	N_{area}	C: N	St/N
Low	Intercept	−20.168	1.900	−2.474	73.227	5.89×10^8
	Slope	0.007	0.004	0.002	−0.013	−140000
	P	0.002	0.006	<0.001	0.008	<0.001
High	Intercept	19.918	44.484	6.704	−37.036	6.22×10^6
	Slope	−0.006	−0.009	−0.001	0.022	50 000
	P	0.426	0.210	0.315	0.309	0.551

$\delta^{15}N$, ratio of ^{15}N to ^{14}N; N_{mass}, N_{area}, leaf N concentration per unit mass and area; C: N, ratio of leaf N concentration to leaf C concentration; St/N (10^5 g^{-1} N), number of stomata per gram of leaf nitrogen and mass.

Table 2: regression equations for foliar $\delta^{13}C$ values (‰) against other physiological leaf traits in *Quercus aquifolioides*.

Leaf traits	Statistic			
	Intercept	Slope	R^2	P
LMA	−33.691	0.358	0.740	<0.001
C_{mass}	54.509	−0.166	0.300	0.053
C_{area}	−33.771	0.073	0.731	<0.001
SD	−12.365	−0.039	0.498	0.007
SL	−10.724	−1.069	0.531	0.005
St/LM	−22.947	-1.96×10^{-6}	0.712	<0.001
LA	−25.317	−0.165	0.234	0.094
LM	−30.990	1.4126	0.501	0.007
LT	−32.363	17.681	0.655	0.001

LMA (mg·cm⁻²), leaf mass per unit area; C_{mass} (mg·g⁻¹), C_{area} (g·m⁻²), leaf C concentration per unit mass and area; SD (mm⁻²), stomatal density; SL (µm), the length of stomata; St/LM (10^5 g^{-1}), number of stomata per leaf mass; LA (cm²), leaf area; LM (g), leaf mass; LT (mm), leaf thickness.

Table 3: regression equations for foliar $\delta^{15}N$ (‰) values against other physiological leaf traits in *Quercus aquifolioides*.

Leaf traits	Statistic			
	Intercept	Slope	R^2	P
N_{mass}	−9.376	0.677	0.581	0.002
N_{area}	−10.952	4.782	0.723	<0.001
C: N	10.158	−0.273	0.722	<0.001
St/N	9.991	-5.70×10^{-8}	0.618	0.001

N_{mass} (mg g⁻¹), N_{area} (g m⁻²), leaf N concentration per unit mass and area; C:N, ratio of leaf C concentration to leaf N concentration; St/N (10^5 g^{-1} N), number of stomata per gram of leaf nitrogen.

relationships with C:N and St/N, suggesting that altitudinal variations in $\delta^{15}N$ may be closely linked to soil nutrient status, particularly soil N availability.

Ecological consequences of altitudinal differences in foliar $\delta^{13}C$, $\delta^{15}N$ and other leaf traits

The 11 leaf traits were examined using PCA. The first PCA axis accounted for 47.00% of the total variation across the altitudinal gradient, compared with 27.24% for the second axis (Fig. 4). Out of all the traits, $\delta^{13}C$, LMA, C_{mass}, LT, SD, SL and St/LM contributed substantially to the first axis, while $\delta^{15}N$, N_{mass}, C:N and St/N contributed substantially to the second axis. Increasing scores on the first axis were associated with altitude; variables linked to water conservation, i.e. $\delta^{13}C$, LMA and LT also increased while SD, SL and St/LM declined (Fig. 4). On the second PCA axis increasing scores were also associated with increasing altitude; the variables associated with this axis reflected the plant's nitrogen acquisition strategy, i.e. $\delta^{15}N$, N_{mass}, C:N, but exhibited nonlinear shifts, with a clear transitional zone at around 3300 m (Fig. 4). In addition, the two axes were orthogonal, suggesting that water and nitrogen use strategies in *Q. aquifolioides* operate independently along the altitudinal gradient.
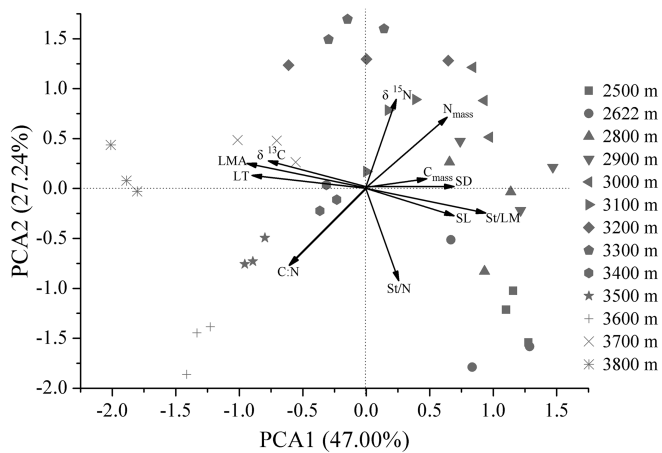
Figure 4: results of the principal component analysis based on the 11 functional traits measured for *Quercus aquifolioides* across a range of altitudes in the Sygera Mountains, southeastern Tibetan Plateau.

DISCUSSION

Altitudinal variation in foliar $\delta^{13}C$

Our results suggest that foliar $\delta^{13}C$ in *Q. aquifolioides* increases with altitude; this is consistent with many previous studies of altitude (Hultine and Marshall 2000; Körner *et al.* 1988, 1991; Marshall and Zhang 1994; Sparks and Ehleringer 1997; Vitousek *et al.* 1990; Warren *et al.* 2001). In general, most leaf N is bound up in photosynthetic enzymes, and thus plants with higher N concentrations in their leaves are considered to have greater photosynthetic capacity (Evans 1989; Field and Mooney 1986). However, leaf N concentrations, particularly per unit area (N_{area}), exhibit an increasing trend with altitude in several species (Cordell *et al.* 1999; Körner 1989; Oleksyn *et al.* 1998; Read *et al.* 2014; Sparks and Ehleringer 1997; Suzuki 1998; Weih and Karlsson 1999). In this study, we found that N_{mass} and N_{area} increased with increasing altitude, but not in a linear fashion, and they were not correlated with foliar $\delta^{13}C$ (Fig. 3). Furthermore, while both St/N and St/LM have previously been found to positively related to leaf CO_2 supply and thus negatively related to foliar $\delta^{13}C$ (Hultine and Marshall 2000), only St/LM was significantly negatively correlated with foliar $\delta^{13}C$ in our study (Table 3). This finding implies that CO_2 supply could cause the altitudinal variation in foliar $\delta^{13}C$ in *Q. aquifolioides*. Similarly, a constant N_{area} value with increasing altitude has been found (Hultine and Marshall 2000), and a reduction in photosynthetic capacity has also been found in other species (Cabrera *et al.* 1998; Rada *et al.* 1998; Zhang *et al.* 2005). Consequently, the variation in the photosynthetic capacity of *Q. aquifolioides* may not be the main cause of the observed $\delta^{13}C$ trend with altitude .

Another possible factor causing foliar $\delta^{13}C$ to increase with altitude is the higher leaf internal resistance associated with thicker leaves (Morecroft *et al.* 1992) and increased LMA, which is an intermediate index of supply and demand for CO_2 (Hultine and Marshall 2000). This is because the

longer diffusive path found in thicker leaves increases internal mesophyll resistance and thus reduces CO_2 concentrations at the site of carboxylation below P_{int}, the partial pressure in the substomatal cavities; such a decline in partial pressure would tend to increase foliar $\delta^{13}C$ (Vitousek *et al.* 1990). Higher internal mesophyll resistance has been validated indirectly and directly in several species (Hultine and Marshall 2000; Kogami *et al.* 2001). Similarly, LA decreased while LM, LT and C_{area} increased significantly with increasing altitude (Fig. 3), thus contributing to the increasing LMA with altitude. Furthermore, strong positive relationships between foliar $\delta^{13}C$ and LT or LMA were found in *Q. aquifolioides* ($R^2 = 0.740$, $P < 0.001$; $R^2 = 0.655$, $P = 0.001$, Table 2). Thus, leaf internal mesophyll resistance related to LMA could be one important factor causing the observed increases in foliar $\delta^{13}C$ in *Q. aquifolioides* across the altitudinal range.

Besides internal mesophyll resistance, increases in foliar $\delta^{13}C$ with altitude have also been attributed to a decrease in maximum foliar stomatal conductance (Meinzer *et al.* 1992; Morecroft *et al.* 1992), presumably determined by stomatal density and size (Franks and Beerling 2009; Woodward and Bazzaz 1988). Like many broad-leaved and coniferous woody species (Hultine and Marshall 2000; Körner *et al.* 1983; Schoettle and Rochelle 2000), SD in combination with stomatal size (as indicated by SL) declined linearly in *Q. aquifolioides*, indicating that stomatal conductance decreased with altitude (Fig. 2). Because plants in alpine regions often encounter low temperatures, high wind speeds and increased vapor pressure deficits between their leaves and the air (Tranquillini 1964), the reduced SD is the result of acclimation to reduce water loss, suggesting this could be more advantageous for water conservation than facilitating carbon gain at dry high-elevation sites (Schoettle and Rochelle 2000). At our study sites, the decline in MAT from 8.6, 1.8 to −0.73°C at altitudes of 3000, 3500, and 3800 m, respectively, also confirmed this view. Taken together, the main cause of the variation in foliar $\delta^{13}C$ in *Q. aquifolioides* with altitude is likely leaf internal resistance and stomatal conductance affected by LMA, LT, SD and SL, rather than photosynthetic capacity as indicated by foliar nitrogen concentration.

Altitudinal variation in foliar $\delta^{15}N$

Foliar $\delta^{15}N$, as an integrator of terrestrial N cycling (Robinson 2001), has been found to have close relationships with various environmental factors (Amundson *et al.* 2003; Craine *et al.* 2009; Handley *et al.* 1999; Martinelli *et al.* 1999). However, there have been very few studies examining the relationship between foliar $\delta^{15}N$ and altitude (Sah and Brume 2003; Virginia and Delwiche 1982; Vitousek *et al.* 1989). Even from these few studies, there are conflicting results. For example, foliar $\delta^{15}N$ exhibited a decreasing trend with altitude in plants collected along gradients across California (Virginia and Delwiche 1982) and the Kathmandu valley in Nepal (Sah and Brume 2003), whereas in the tree *Metrosiderous*, studied near

the Hawaiian coast, it exhibited no differences with altitude (Vitousek *et al.* 1989). In contrast to these results, we found that foliar $\delta^{15}N$ in *Q. aquifolioides* showed a nonlinear trend with altitude: initially increasing significantly, and then declining (Fig. 3A). On the eastern slopes of the Sygera Mountains, MAP increases linearly while MAT declines from an altitude of 3000 m up to 3800 m. Foliar $\delta^{15}N$ is positively correlated with MAT (when MAT $\geq -0.5°C$) and negatively correlated with MAP at both regional and global scales (Craine *et al.* 2009; Handley *et al.* 1999), so the changes in MAP and MAT are unlikely to explain the nonlinear changes in foliar $\delta^{15}N$ in *Q. aquifolioides* with altitude.

Since foliar N concentrations have been used broadly as an index of soil N availability (Asner and Vitousek 2005; Ollinger *et al.* 2002; Ordoñez *et al.* 2009; Reich *et al.* 2001; Xia and Wan 2008), and further $\delta^{15}N$ is positively associated with terrestrial soil N availability on local to regional scales (Craine *et al.* 2009; Garten *et al.* 2011; Högberg 1997; McLauchlan *et al.* 2010), our data indicated that foliar $\delta^{15}N$ had a strong positive relationship with leaf N concentrations (both N_{mass} and N_{area}), highlighting that the changing pattern of foliar $\delta^{15}N$ with altitude may be closely associated with soil N availability. With increasing soil N availability, the production of ammonium, and subsequently nitrate, increases. Gaseous N loss during nitrification (Firestone and Davidson 1989) and the leaching of ^{15}N-depleted nitrate (Högberg 1997; Koba *et al.* 2003) can lead to the remaining N pool (and subsequently the levels in plants) to be enriched in ^{15}N. As N availability further increases, denitrificaiton begins to consume a larger proportion of the nitrate present (Hall and Matson 2003), further enriching inorganic N pools if denitrification does not completely consume the nitrate pool (Houlton *et al.* 2006). Although our data did not confirm these processes in detail due to lack of data on soil properties, i.e. soil $\delta^{15}N$, soil N content, and soil NH_4^--N and NO_3^--N content, they did indicate that foliar $\delta^{15}N$ is closely related to soil N availability in *Q. aquifolioides* across the altitudinal range studied. Furthermore, there is much evidence that the natural abundance of ^{15}N is greater in systems where nutrients are not limiting (Craine *et al.* 2009). Thus, higher foliar $\delta^{15}N$ values appear to indicate a more open N cycle, with greater losses relative to turnover. If this is true, the varying pattern of foliar $\delta^{15}N$ in our study suggests that the sites at around 3300 m might have a more open N cycle relative to the low- and high-altitudes occupied by *Q. aquifolioides*. In addition, compared to the decrease at high elevations (3400, 3500 and 3600 m), foliar $\delta^{15}N$ slightly increased again at the highest elevations of 3700 and 3800 m, which have relatively higher precipitation. Since preferential leaching of isotopically light N into stream water (Amundson *et al.* 2003; Austin and Vitousek 1998; Högberg 1997; Vitousek 2002), we speculated that isotope discrimination of hydraulic leaching may be responsible for this slightly increasing change. To much better understanding the mechanisms underlying the pattern of foliar $\delta^{15}N$ and nitrogen cycle

across the altitudinal gradient in alpine regions, future studies are needed to quantify the effects of soil and climatic factors on foliar $\delta^{15}N$ at multiple species and even community levels on alpine regions.

Intraspecific trade-offs in foliar $\delta^{13}C$ and $\delta^{15}N$ with other leaf traits at different altitudes

It is now widely accepted that, in vascular plants, there is a fundamental trade-off between rapid acquisition and conservation of resources (Grime *et al.* 1997; Reich *et al.* 1997; Wright *et al.* 2004), described as the 'leaf economics spectrum' (Wright *et al.* 2004); this is mostly based on comparisons between species. However, to date, only one study (Albert *et al.* 2010) has demonstrated that such relationships also occur within a species. Here we have shown that the same trade-off exists in *Q. aquifolioides* along a wide altitudinal gradient on the Tibetan Plateau. PCA identified one axis representing a water availability gradient from the low-altitude populations with lower $\delta^{13}C$, LMA and LT and higher SD, SL and St/LM, to the high-altitude populations with higher $\delta^{13}C$, LMA, and LT and lower SD, SL and St/LM (Fig. 4). Many environmental stimuli cause plants to regulate water loss and photosynthetic carbon gain, both dynamically by the opening and closing of stomata, and structurally by growing leaves with altered stomatal densities and pore sizes (Brodribb and McAdam 2011; de Boer *et al.* 2011). With the progressively declining water availability associated with low air and soil temperatures, high wind speeds and increased vapor pressure deficit between leaves and the ambient air in alpine regions, plants are able to enhance water use efficiency ($\delta^{13}C$) and LMA, and reduce stomatal density and size in order to optimize intraspecific water availability. Therefore, the first axis of our PCA reflected the intraspecific trade-off between water loss and photosynthetic carbon gain, and consequently the trade-off between persistence and growth in *Q. aquifolioides* across altitudes.

In contrast to the first axis, the second axis reflected a nitrogen use strategy in which the mid-altitude populations had higher foliar $\delta^{15}N$, N_{mass} and lower C:N, while the low- and high-altitude populations had lower $\delta^{15}N$, N_{mass} and higher C:N (Fig. 4). Although the N cycle is complex, the combination of foliar $\delta^{15}N$ and N concentrations could strongly reflect terrestrial soil N availability from local to global scales in the above-mentioned many studies. The shifting patterns associated with foliar $\delta^{15}N$, N_{mass} and C:N in this study indicated that the mid-altitude populations experienced higher soil N availability, while the low- and high-altitude populations experienced lower soil N availability and in these populations the underlying metabolic processes are different. At altitudes above 3300 m, the reduced N availability could be caused by the very low MAT, which suppressed not only the decomposition and mineralization of organic matter, but also uptake of nutrients by roots. This is in line with the biogeochemical hypothesis (Reich *et al.* 2004). In contrast, at altitudes below 3300 m, there was low

N availability; here, high leaf N relative to C at low MATs might arise as increasing N offsets the reduced efficiency of enzymes and physiological processes. This is in line with the temperature-physiological hypothesis (Reich and Oleksyn 2004). From the N availability perspective, therefore, the mid-altitudes around 3300 m may be the optimum zone for the growth of *Q. aquifolioides*.

CONCLUSIONS

Contrary to our hypothesis, our results indicated that foliar $\delta^{13}C$ and $\delta^{15}N$ in *Q. aquifolioides* exhibited different patterns along the altitudinal gradient on the Tibetan Plateau. Further, Variation in foliar $\delta^{13}C$, along with LMA, LM, LT, SD, SL and St/LM, may reflect trade-off between water loss and carbon gain. In contrast, variation in foliar $\delta^{15}N$, along with N_{mass} and C:N, may reflect nitrogen acquisition strategy in *Q. aquifolioides* across its altitudinal distribution.

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