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Functional traits related to environmental divergence in combination with phylogenetic relationship of *Picea* species

Received: 1 September 2018; Accepted: 22 January 2019

Abstract: Background: Plants have adapted to fine-scale environmental heterogeneity through ecologically important traits, leading to new lineages. This suggests that differentiation of important traits has been beneficial to habitat partitioning among closely related species. However, the pattern of ecological divergences and differentiation of functional traits in combination with phylogenetic relationships have not been widely examined.

Material and methods: To illustrate the pattern of ecological divergences of nine *Picea* species, spatial evolutionary and ecological vicariance analysis (seeva) was used to quantify and test the divergence between sister lineages. Five functional traits were measured in a common garden experiment. For all traits the quantitative convergence index (QVI) was determined. Furthermore, their relationships with divergent environmental variables were analyzed in the context of phylogenetic relationships.

Results: Temperature variables (maximum temperature of warmest month and temperature annual range) split at basal nodes and precipitation variables (annual precipitation and precipitation of driest quarter) split at terminal nodes. Conservative traits were leaf mass per area (LMA) and net photosynthetic rate (Pn), which reflected selective retention when ancestors suffered cold environmental separation. Meanwhile, linear stomatal density (LSD), carbon-13 isotope ratio ($\delta^{13}\text{C}$) and water potential at 50% loss of hydraulic conductivity (P_{50}) were convergent between species, but only P_{50} exhibited adaption to different precipitation conditions.

The nine *Picea* species exhibit a distinct environmental divergence pattern. LMA and Pn were selectively retained when their ancestors were subjected to cold environmental separation. P_{50} was an important trait with respect to adaptation to precipitation differences. This research provided a new way of expounding the correlation between environment, functional traits and phylogeny, deepening our understanding of environmental divergence, trait differentiation and speciation.

Keywords: $\delta^{13}\text{C}$, P_{50} , *Picea*, seeva, quantitative convergence index

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Introduction

Divergent natural selection based on ecologically important traits leads to different adaptations, evolutionary divergence, and reproductive isolation between incipient species (Wiens, 2004). Furthermore, it may be common in plant species because of its sensitivity to fine-scale environmental heterogeneity (Givnish, 2010; Anacker & Strauss, 2014). Several studies have evaluated that occupation of novel habitats and development of drastic ecological shifts have led to new lineages and species through adaptive radiation and acquisition of new genetic traits; thus, current habitat preferences could be expected to reflect ancestral preferences to a first approximation (Peterson et al., 1999; Webb et al., 2002; Wiens & Graham, 2005; Rundell & Price, 2009). Generally, researchers have used phylogeographic methods to illustrate species evolution or mapped ecological traits onto phylogenies to provide insights into ecological vicariance and/or ancestral ecological traits (e.g. Hardy & Linder, 2005; Zhang et al., 2011; Pittermann et al., 2012; Schulte et al., 2015). They have seldom taken environmental and physiological data into account simultaneously. Therefore, as a novel approach to biogeography and speciation research, Struwe et al. (2011) suggested spatial evolutionary and ecological vicariance analysis (seeva) to quantify and test the divergence between sister lineages and illustrate both niche conservatism and ecological specialization. Seeva yields an analysis of directly observed data from individuals, avoiding the necessary loss of information that accompanies traditional averaging and providing us with an opportunity to understand the interaction between species and their habitat selection during evolution (Qu et al., 2015; Ye et al., 2016).

It is well known that adaptation of plants to their environment occurs through several important (adaptive or functional) traits (Flatscher et al., 2012). Stomatal density plays an important role in the interaction between plants and environment (Brodrribb et al., 2009, 2014; Casson & Hetherington, 2010; Drake et al., 2013), and it has been predicted that stomata are important contributors to speciation and evolutionary change (Hetherington & Woodward, 2003; Franks & Berling, 2009; Tiwari et al., 2013). For example, plants with high stomatal density in relatively arid sites also have higher stomatal conductance than those with low stomatal density, because their intrinsically higher rates of gas exchange increased capacity for transpirational cooling and more rapid stomatal closure in response to drought stress (Franks et al., 2009; Drake et al., 2013). Other important traits are selected because of its relationship with physiological activities. Species

with smaller and thicker leaves (high LMA) generally have lower mass-based rates of photosynthesis (Pn-mass) (Han, 2011; Coble & Cavaleri, 2014). Besides, physiological traits usually reflect the actual function of plants, i.e. adaption to habitat. Hydraulic parameters, such as P_{50} (water potential at 50% loss of hydraulic conductivity), is widely used to indicate the resistance of plants to xylem hydraulic failure across species or due to environmental change (Iovi et al., 2009; Vergeynst et al., 2015). Species from dry or cold environment always have a lower P_{50} in order to resist drought-induced or freeze-induced cavitation (Pockman & Sperry, 2000; Pittermann & Sperry, 2003; Cobb et al., 2007; Gea-Izquierdo et al., 2012). Previous research paid more attentions to the correlations among functional traits and between traits and the environment or examined whether these relationships are genuine or not through phylogenetic analysis (Willson et al., 2008; Brodrribb et al., 2009; Drake et al., 2013; Aguilarromero et al., 2017). Functional traits and environmental divergence of species have rarely been discussed in relation to phylogenetic background.

Picea (spruce) species, which are important components of the alpine and sub-alpine forests in the Northern Hemisphere, are classified morphologically in terms of their needle shape, number of stomatal rows, stomata arrangement, number and position, and cone volume (Fu et al., 1999; Ghimire et al., 2014, 2015). Molecular evidence indicates that *Picea* speciation reflects recent adaptive radiation (Ran et al., 2006; Lockwood et al., 2013), whilst, the continuous and rapid uplift of the Qinghai-Tibet Plateau (QTP) has affected the climate and the environment of China (Zhang et al., 2000). Hence, spruces represent a good model for studying historical ecological vicariance and examining the differentiation of functional traits. We used a common garden experiment to standardize plants' growing environment (Lewis et al., 2011; Pittermann et al., 2012) and then assessed environmental divergence and differentiation of five important traits (LSD: linear stomatal density, LMA: leaf dry-mass per area, Pn: net photosynthetic rate, $\delta^{13}\text{C}$: carbon-13 isotope ratio and P_{50} : water potential at 50% loss of hydraulic conductivity) of nine *Picea* taxa, which covered plants originating from across a wide gradient of precipitation and temperature, from the southwest to the northeast of China. Three general questions were considered. (1) What pattern do ecological divergences present between sister lineages in the phylogenetic context of *Picea*? (2) What difference of these traits and which display conservatism/convergence in the common garden? (3) What do the conservative/convergent traits indicate in terms of the relationship between environmental variables and traits?

Materials and methods

Plant materials and geolocation

Spruces were planted in the Plant Germplasm Repository of Lanzhou University, located on Yuzhong campus, China (35°56'37"N, 104°09'05"E, Alt: 1750 m above mean sea level). The site is characterized by a continental semi-arid climate with strong seasonality. The annual mean temperature is 7.1°C and mean growing-season temperature is 13°C. Mean annual precipitation is 385 mm with more than half of the annual total falling during the growing season (c. 218 mm) (Zhang et al., 2015). Seeds of *Picea* taxa from each distribution range were collected over ten years ago from at least one population. All seeds were germinated at a tree nursery in 2005. Five years later, all seedlings were successively transplanted outdoors in spring of 2010 and were studied in mid-September 2013. The common garden plantation contains four blocks (replicates), one plot positioned within each block. Each plot was initially represented by nine trees of the same provenance by a 3 × 3 arrangement, planted one meter apart. Each tree was watered by a scheduled irrigation system to ensure sufficient water. Nine *Picea* species were selected, named *Picea. crassifolia*, *P. meyeri*, *P. asperata*, *P. koraiensis*, *P. wilsonii*, *P. spinulosa*, *P. likiangensis* vars. *likiangensis*, *linzhiensis* and *rubescens*, which exhibited obvious geographical vicarism in China (Table 1).

To obtain general information related to the occurrence of the *Picea* species and to facilitate representative sampling of occurrence sites from their

entire ranges, we searched for information from our laboratory sampling records and the Chinese Virtual Herbarium (CVH; <http://www.cvh.org.cn/english-index.asp>) database. Simultaneously, we coded data quality for each collection record (especially when exact geo-coordinates data were lacking) according to the degree of precision for each pair of latitude and longitude coordinates and deleted repeated data. In the end, 323 collections were retained (data range is presented in Table 1). To ensure accuracy of the locations, latitude and longitude decimal degrees were used as coordinates, which were converted to a point shapefile (Fig. 1) in ArcGIS10.0 (Environmental Systems research, Redlands, CA, USA).

Environmental variables

Local environmental variables were extracted from eight layers in ArcGIS with our records of collection point locations and acquired primary GIS data layers for temperature and precipitation data from Worldclim v. 1.4 (<http://www.worldclim.org>; Hijmans et al., 2005) according to Struwe et al. (2011), including annual mean temperature (°C), maximum temperature of warmest month (°C), minimum temperature of coldest month (°C), annual temperature range (°C), annual precipitation (mm), precipitation of wettest month (mm), precipitation of wettest quarter (mm), and precipitation of driest quarter (mm) (Table 1). Then the environmental data from ArcGIS were saved as an excel worksheet for use in subsequent seeva statistical analysis (Table 2).

Table 1. Range of longitude and latitude of distribution and mean (\pm standard error) environmental variables. Collection sites were selected based on our laboratory records and Chinese Virtual Herbarium (CVH) records

Taxa	Longitude (E)	Latitude (N)	MAT (°C)	MTWM (°C)	MTCM (°C)	TAR (°C)	MAP (mm)	PWM (mm)	PWQ (mm)	PDQ (mm)
<i>Picea. crassifolia</i>	100.9–104.3	33.6–37.8	3.66 ± 0.29 a	20.17 ± 0.31 b	–16.26 ± 0.37 c	36.44 ± 0.28 d	500.56 ± 14.53 a	102.24 ± 1.74 a	275.03 ± 5.69 a	9.32 ± 0.44 a
<i>Picea. asperata</i>	110.3–111.1	37.3–44.0	5.24 ± 0.30 b	20.40 ± 0.27 b	–13.95 ± 0.46 d	34.35 ± 0.35 c	700.61 ± 14.01 b	127.79 ± 2.65 b	353.70 ± 6.54 b	15.67 ± 0.61 ab
<i>Picea. meyeri</i>	101.1–104.4	30.3–37.8	5.11 ± 0.56 b	25.76 ± 0.57 c	–18.40 ± 0.68 b	44.16 ± 0.61 f	457.48 ± 15.67 a	127.26 ± 3.69 b	299.65 ± 8.78 a	12.13 ± 0.84 ab
<i>Picea. koraiensis</i>	126.5–130.5	41.4–47.0	2.73 ± 0.18 a	25.75 ± 0.24 c	–24.85 ± 0.36 a	50.61 ± 0.48 g	656.04 ± 16.08 b	159.92 ± 4.37 c	407.54 ± 9.81 c	23.50 ± 1.19 c
<i>Picea. wilsonii</i>	101.6–116.8	33.7–40.8	7.67 ± 0.43 c	25.44 ± 0.52 c	–12.47 ± 0.66 d	37.91 ± 0.75 e	627.75 ± 40.47 b	136.60 ± 5.79 b	342.87 ± 14.50 b	22.96 ± 3.33 c
<i>Picea. spinulosa</i>	88.5–92.0	27.2–30.3	9.49 ± 0.22 d	20.62 ± 0.26 b	–6.42 ± 0.19 f	27.04 ± 0.22 a	1039.10 ± 23.42 d	229.04 ± 4.84 e	618.04 ± 13.40 e	18.67 ± 1.51 bc
<i>Picea. likiangensis</i> var. <i>likiangensis</i>	98.8–101.9	26.8–30.0	7.05 ± 0.23 c	18.24 ± 0.20 a	–8.72 ± 0.34 e	26.96 ± 0.29 a	835.90 ± 15.47 c	200.31 ± 3.98 d	518.48 ± 10.30 d	15.86 ± 0.89 ab
<i>Picea. likiangensis</i> var. <i>rubescens</i>	94.4–103.0	28.3–32.9	4.68 ± 0.20 b	18.72 ± 0.24 a	–13.26 ± 0.29 d	31.97 ± 0.38 b	644.23 ± 18.53 b	139.49 ± 3.32 b	380.74 ± 8.78 bc	12.10 ± 0.79 ab
<i>Picea. likiangensis</i> var. <i>linzhiensis</i>	92.9–100.6	27.8–30.3	7.70 ± 0.24 c	19.94 ± 0.30 b	–8.05 ± 0.32 e	28.00 ± 0.35 a	692.37 ± 14.75 b	154.33 ± 3.84 c	412.20 ± 8.85 c	14.60 ± 1.33 ab

Letters (a, b, c, etc) after the numbers distinguish between statistically different values ($P < 0.01$) for different taxa.

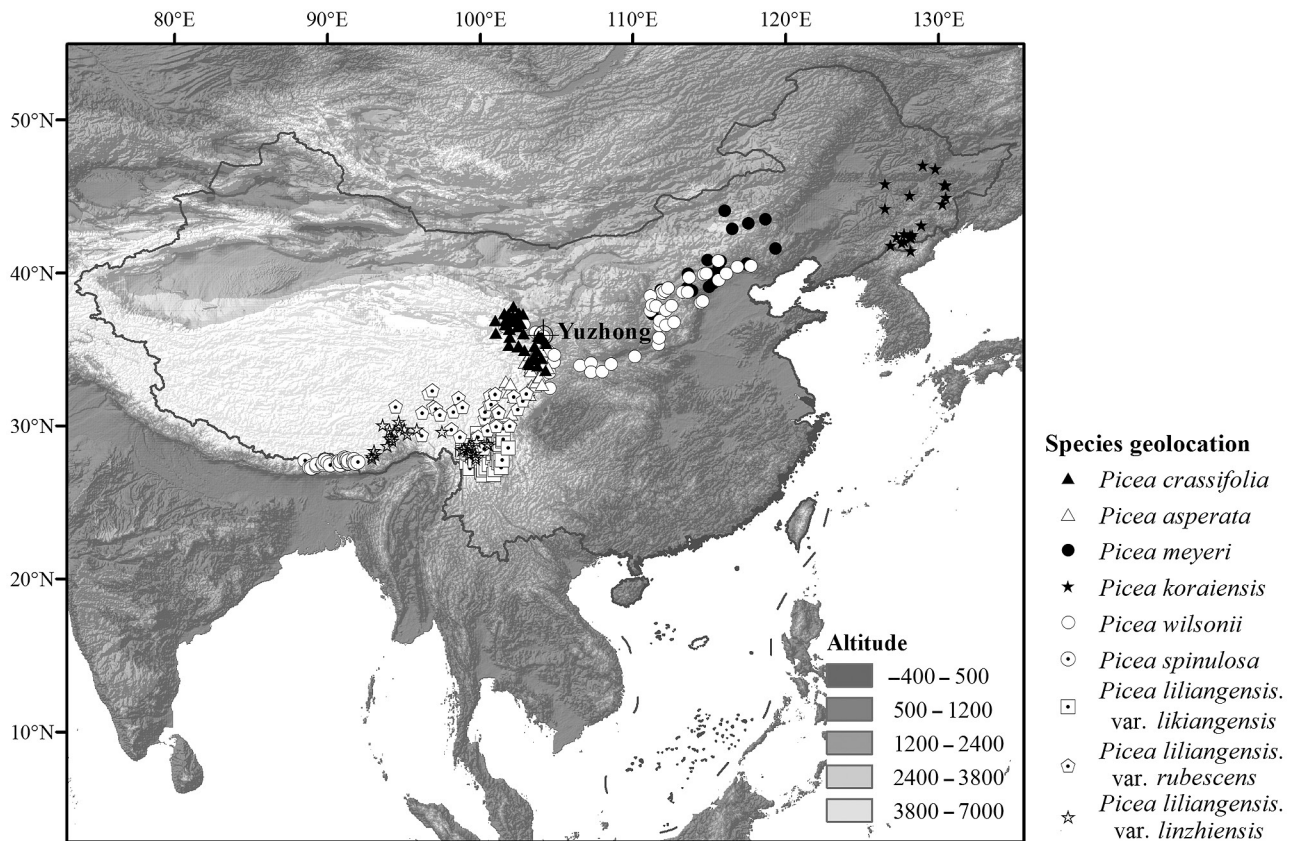


Fig. 1. Geographic location of nine *Picea* species

Phylogenetic data and seeva analysis

According to the phylogenetic relationships of *Picea* reconstructed by Lockwood et al. (2013), we used Tree View software (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>) to rebuild a phylogenetic tree for these nine taxa. A Nexus-format tree file was then exported from the software and analyzed in seevav. 1.01. Data of species divergence time were collected from previous results (see Table S1 in the Supplementary Data) and mapped in the phylogenetic tree.

To detect ecological divergence among sister lineages in the phylogenetic tree, we used the spatial evolutionary and ecological vicariance analysis (seeva, Struwe et al., 2011). The seeva can investigate ecological vicariance in speciation by statistical methods, which incorporates environmental data with phylogenetic data. It ends up with the differences between each of the environmental variables for each node. The differences are reflected by a divergence index (D) with the range 0 to 1, which was calculated for each environmental factor at each node. $D = 1$ indicates a maximum difference between sister clades, whereas $D = 0$ indicates no difference. To determine the significance of D , a Bonferroni correction was performed by the seeva. Because eight independent tests were conducted for each of the

environmental variables, a P -value less than 0.0064 ($\alpha = 1 - 0.95^{1/8} = 0.006391$) of D indicated a significant difference in the ecological features for splits at a given node.

Linear stomatal density

Three individuals per species were randomly chosen from three different plots for anatomical measurements in mid-September 2013. On a sunny morning, sunny side of current-year branches were cut down and placed in a 10ml centrifuge tube with modified FAA solution (50% alcohol, formalin, glacial acetic acid and glycerin volume ratio of 90 ml: 5 ml: 5 ml: 5 ml) immediately to prevent stomatal closure. All samples were brought back to the laboratory for measurement of anatomical characteristics. Twenty needles from each branch were randomly selected and sliced the mid-arrise before mesophyll tissue being dissociated using sodium hypochlorite. Banister brush was used to clean the residual mesophyll tissue after dissociation. The leaves were stained with safranin to make temporary slide and then pictures were taken using an Olympus photomicroscope (Olympus (China) CO., LTD.) at $\times 40$ magnification. Linear stomatal density (LSD, mm^{-1}) was calculated as described by Zhao et al. (2008), the sum of the mean number of stomata per 1 mm

length on the leaf surface. To avoid variations at the base and tip, stomatal counts on the epidermis were conducted near the middle of the needle using Image-Pro Plus 5.0.1 (Media Cybernetics, Inc., USA) software.

Net photosynthesis rate and leaf mass per area

The net photosynthesis rate was measured under 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light-level (saturate red and blue light) for fully expanded current-year needles in a LI-6400XT gas exchange system (LI-COR Biosciences, Lincoln, NE) with the 6400-22L conifer chamber. At least three random seedlings for each treatment per species were measured. All needles were scanned to measure their projected areas and then dried in an oven at 72°C to allow us to calculate net photosynthesis rate per mass (Pn) and leaf mass per area (LMA).

Carbon isotope ratio

Needles were collected from the same plants for which net photosynthesis rate had been measured, for stable carbon isotope ($\delta^{13}\text{C}$) analysis. These needles were rinsed with deionized water to remove soil and then dried in an oven at 72°C. The dried foliage was then separated and ground to a fine powder. Approximately 2 mg of powder was weighed and sealed in silver paper, then combusted in a vario TOC (Elementar Trading (Shanghai) Co., Ltd). Combustion products were swept by an equilibrium gas and continuous-flow interface into a carbon dioxide isotope analyzer CCIA-36d (LGR, Inc., San Jose, CA). The carbon-13 isotope ratio was calculated as follows:

$$\delta^{13}\text{C}(\text{‰}) = \frac{(^{13}\text{C}/^{12}\text{C})_L - (^{13}\text{C}/^{12}\text{C})_S}{(^{13}\text{C}/^{12}\text{C})_S} \times 1000$$

where $(^{13}\text{C}/^{12}\text{C})_L$ and $(^{13}\text{C}/^{12}\text{C})_S$ are the $^{13}\text{C}/^{12}\text{C}$ value of leaves and PDB (standard substance), respectively.

Water potential at 50% loss of hydraulic conductivity

One-year branches were selected at half tree height from each individual early in the morning. Then basal sections were immediately recut under water and transported to the laboratory in opaque plastic bags. All branches were recut again to remove any pine oil before being immersed in water for fully hydration. The hydraulic conductivity and water potential of

each tree were measured by a modified flush method and a pressure chamber method, respectively (Sperry et al., 1988; Sack et al., 2002). Then vulnerability curves were constructed and fitted using an exponential sigmoidal equation:

$$K = \frac{100}{1 + e^{-\frac{(P - P_{50})}{a}}}$$

where K is percent loss of hydraulic conductivity, P is water potential at relevant K, P_{50} is water potential at 50% loss of hydraulic conductivity and 'a' is reciprocal value of the relative slope of the curve.

Convergence index

The quantitative convergence index (QVI) was used to quantify levels of convergent evolution for each trait; this was developed by Ackerly and Donoghue (1998) for continuous characters. This method incorporates the phylogenetic relationship among species to evaluate the evolutionary patterns underlying present-day trait distributions. QVI ranges from 0, for traits in which closely related species are phenotypically similar; to 1, for traits in which similar species are distantly related and convergent evolution is maximized (Ackerly & Reich, 1999; Couvreur et al., 2011). Due to the certain phylogenetic relationship, the calculation of QVI was just undertaken with 1000 randomizations of the tree tips using the software program CACTUS 1.13 (Schwilk & Ackerly, 2001; Willson et al., 2008).

Statistical analysis

First, the significance of all differences in traits and environmental variables between *Picea* taxa at the 5% level were determined by one-way analyses of variance (ANOVA) and LSD tests for multiple comparisons, in addition to principal component analysis (PCA) for environmental variables, using SPSS 16.0 (SPSS Inc., Chicago, IL). Second, we tested the ecological divergences under the phylogenetic background through seeva (download link is <http://seeva.heiberg.se>; Struwe et al., 2011). Finally, relationships between morphological and physiological traits and environmental variables were plotted by SigmaPlot 10.0 (Systat software, Inc.).

Results

Ecological and evolutionary integration

Seeva was used to compare the eight phylogenetic nodes for each of the eight environmental

Table 2. Index of divergence (*D*) from phylogeny-based seeva evaluation of *Picea* taxa, using four temperature-based and four precipitation-based features

Phylogenetic node	Index of divergence (<i>D</i>)							
	MAT (°C)	MTWM (°C)	MTCM (°C)	TAR (°C)	MAP (mm)	PWM (mm)	PWQ (mm)	PDQ (mm)
node1	0.47*	0.17*	0.60*	0.62*	0.31*	0.37*	0.41*	0.01
node2	0.15*	0.88*	0.40*	0.93*	0.01	0.56*	0.11	0.24*
node3	0.20*	0.04	0.19*	0.03	0.69*	0.41*	0.71*	0.76*
node4	0.40*	0.06	0.08	0.00	0.79*	0.29*	0.70*	0.79*
node5	0.01	0.79*	0.24*	0.71*	0.55*	0.41*	0.56*	0.03
node6	0.52*	0.38*	0.50*	0.23*	0.46*	0.41*	0.46*	0.18
node7	0.13	0.13*	0.15*	0.32*	0.47*	0.58*	0.55*	0.22*
node8	0.57*	0.19	0.57*	0.31*	0.18	0.05	0.06	0.06

Features with the larger of the two *D*-values are bolded.

*Nodes with significant differences between sister groups using a Bonferroni correction of $P < 0.0064$.

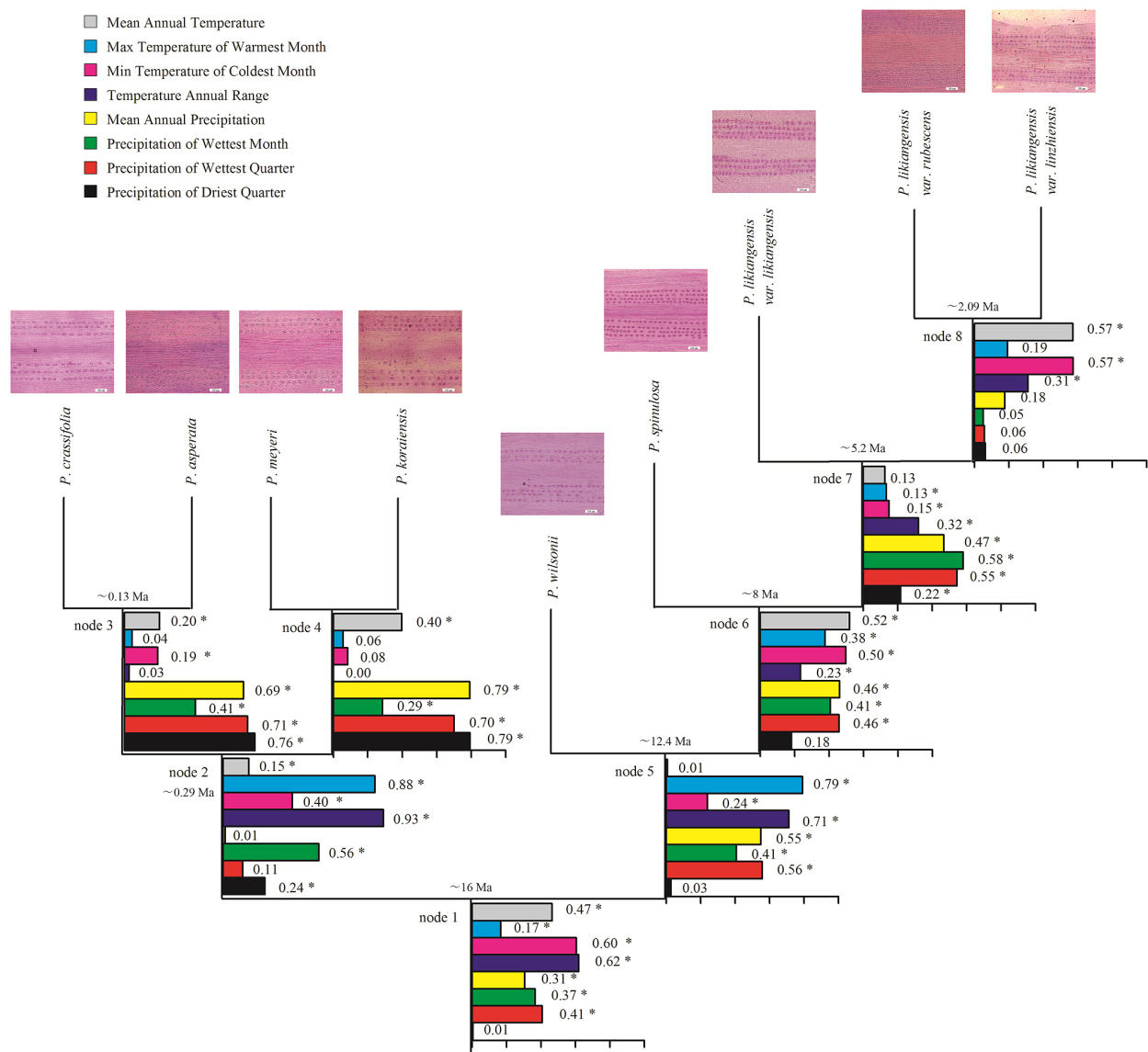


Fig. 2. Hypothetical phylogenetic evolutionary tree for *Picea* taxa, based on Lockwood et al. (2013; outgroups and other taxa excluded). Pictures are part of stomatal anatomy of each species. Divergence indices for eight environmental variables of the phylogeny of *Picea* taxa for each node, shown as histograms from 0 to 1

* Statistically significant divergence indices after Bonferroni correction ($P \leq 0.0064$), showing which variables were significantly different between the sister clades derived from each nodal split.

Table 3. Differences in morphological (LSD and LMA) and physiological traits (Pn, $\delta^{13}\text{C}$ and P_{50}) among *Picea* taxa

Species	LSD (mm)	LMA (kg m ⁻²)	Pn ($\mu\text{mol kg}^{-1}\text{s}^{-1}$)	$\delta^{13}\text{C}$	P_{50} (MPa)
<i>Picea. crassifolia</i>	123.81±3.68 b	0.264±0.016 a	29.98±1.58 e	-23.21±0.21 a	-3.21±0.03 e
<i>Picea. asperata</i>	111.93±3.95 c	0.237±0.016 a	40.61±2.43 cd	-26.38±0.97 cd	-2.71±0.05 d
<i>Picea. meyeri</i>	137.56±4.97 a	0.246±0.016 a	39.49±1.26 cd	-24.94±0.85 abc	-2.83±0.32 de
<i>Picea. koraiensis</i>	130.33±4.83 ab	0.241±0.013 a	37.03±1.13 d	-25.38±0.46 bcd	-2.63±0.31 cd
<i>Picea. wilsonii</i>	74.65±2.74 d	0.158±0.008 cd	58.79±2.48 a	-27.14±0.92 d	-3.18±0.37 de
<i>Picea. spinulosa</i>	108.57±3.90 c	0.131±0.004 d	45.57±0.59 bc	-26.29±0.50 cd	-0.58±0.03 a
<i>Picea. likiangensis. var. likiangensis</i>	134.64±3.95 ab	0.185±0.014 bc	51.27±2.27 b	-23.58±0.34 ab	-1.77±0.05 b
<i>Picea. likiangensis. var. rubescens</i>	129.83±3.40 ab	0.227±0.024 ab	45.12±3.58 bc	-26.12±0.88 cd	-2.66±0.04 d
<i>Picea. likiangensis. var. linzhiensis</i>	84.14±3.00 d	0.177±0.005 c	43.82±2.31 bc	-23.95±0.19 ab	-2.37±0.05 c

Data are presented as mean ± standard error.

Letters (a, b, c, etc) after the numbers distinguish between statistically different values ($P < 0.01$) for different species.

variables; 47 of the 64 comparisons were significant ($P \leq 0.0064$, indicated by *) after Bonferroni correction (Table 2). The complex interaction between environmental variables and the phylogenetic of a particular taxonomic group requires a comparison of D -values between nodes, between variables, and with respect to their relative (temporal) nodal position in the phylogeny. These are exemplified in Fig. 2, which illustrates an overall pattern of ecological vicariance throughout the history of *Picea* species. There is large-scale ecological vicariance at all nodes except 6, 7 and 8, the most recently split in sect. *Casicta* Mayr (*Picea. linkiangnesis* var. *likiangensis*, *P. linkiangnesis* var. *rubescens*, *P. linkiangnesis* var. *linzhiensis*) and *P. spinulosa* shows slight divergence ($D < 0.6$). It is noteworthy that each node shows divergence for different combinations of environmental variables. The consistent divergence at the basal nodes (1, 2,

5) is related to maximum temperature of warmest month and minimum temperature of coldest month, but there is strong divergence with respect to the precipitation variables at the terminal nodes (3, 4). There are some average trends, but the details matter for each node.

For the temperature variables (Table 2), nodes 1, 2, 5 appear to be associated with strong divergence in temperature ($D_2 = 0.88 *$ and $D_5 = 0.79 *$ with respect to maximum temperature of the warmest month; $D_2 = 0.93 *$ and $D_5 = 0.71 *$ with respect to temperature annual range). This means that cool and warm temperature divergence are the main factors that affecting the 2 and 5 nodes' lineages, resulting in them occupying colder environments, which may have led to the differentiation of adaptive traits (Fig. 3, species under node 2 associated colder habitats than those under node 5).

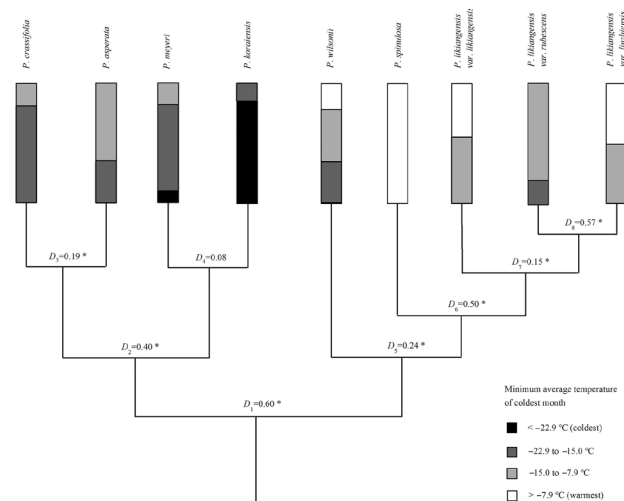


Fig. 3. Seeva results for the variable 'minimum temperature of the coldest month' (coded as four qualitative states) are shown as histograms for each taxa. Total height of each histogram bar equals 100% of observations for each taxa; greyscale colors of histograms represent the four different states. Divergence indices are provided for each node; * $P \leq 0.0064$ (significant difference between clades after Bonferroni correction)

Differentiation of morphological and physiological traits

We measured two morphological traits and three physiological traits in the common garden that enabled us to compare species under uniform environmental conditions and investigate the differentiation between *Picea* species. All traits showed significant differences between these species (Table 3). Linear stomatal density (LSD) ranged from 74.65mm⁻¹ for *P. wilsonii* to 137.56mm⁻¹ for *P. meyeri*. There were no obvious differences in leaf dry-mass per area (LMA) in *P. crassifolia*, *P. asperata*, *P. meyeri* and *P. koraiensis*, which were highest of all. Net photosynthetic rate per dry-mass (Pn) was highest in *P. wilsonii* and lowest in *P. crassifolia*, but the opposite was the case for the carbon-13 isotope ratio ($\delta^{13}\text{C}$), *P. crassifolia* had the highest value (-23.21 ± 0.21) and *P. wilsonii* the lowest (-27.14 ± 0.92). Water potential at 50% loss of hydraulic conductivity (P_{50}) for *P. crassifolia* was also the lowest (-3.21 ± 0.03 MPa), and the highest value was for *P. spinulosa* (-0.58 ± 0.03 MPa).

Correlations between conservative/convergent traits and divergent environment

Values of the quantitative convergence index (QVI) ranged from 0.42 for LMA to 1 for $\delta^{13}\text{C}$ (Table 4). Based on the QVI values, LMA and Pn are conservative and LSD, $\delta^{13}\text{C}$ and P_{50} convergent. In the context of phylogenetic relationship and the seeva results, all species were divided into two groups (group 1: species under node 2; group 2: species under node 5) to test the relationship between conservative traits and divergent environments. LMA decreased from group

Table 4. Quantitative convergence index (QVI) for the five characters

Characters	QVI	Exp QVI	P value
LSD (mm)	0.93	0.81	0.355
LMA (kg m^{-2})	0.42	0.76	0.050
Pn ($\mu\text{mol kg}^{-1} \text{s}^{-1}$)	0.44	0.80	0.045
$\delta^{13}\text{C}$	1.00	0.79	0.000
P_{50} (MPa)	0.80	0.87	0.226

QVI close to 0 indicates closely related species are phenotypically similar; QVI close to 1 indicates that similar species are distantly related and convergent evolution is maximized.

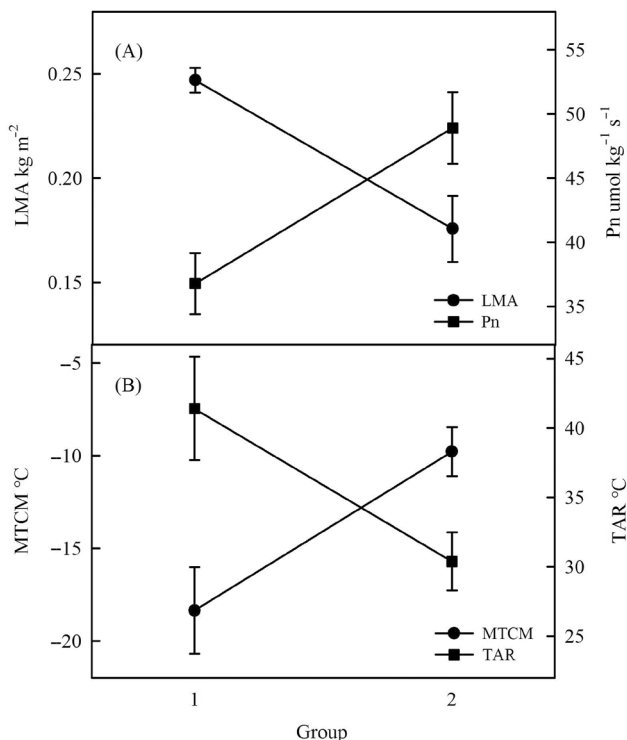


Fig. 4. Variation trends for two conservative traits (a) and two environmental variables (b) between group 1 and 2. Solid circle and square represent leaf dry-mass per area (LMA) and net photosynthetic rate (Pn) in (a), respectively. Solid circle and square represent minimum temperature of coldest month (MTCM) and temperature annual range (TAR) in (b), respectively

Table 5. Relationships between three convergent traits and eight environmental variables as well as their PCA scores

	LSD (mm)	$\delta^{13}\text{C}$	P_{50} (Mpa)
MAT ($^{\circ}\text{C}$)	-0.583 ^m	-0.223	0.653 ^m
MTWM ($^{\circ}\text{C}$)	-0.104	-0.36	-0.368
MTCM ($^{\circ}\text{C}$)	-0.449	0.007	0.592 ^m
TAR ($^{\circ}\text{C}$)	0.279	-0.141	-0.561
MAP (mm)	-0.163	-0.22	0.920
PWM (mm)	0.002	-0.09	0.942 ^{**}
PWQ (mm)	-0.028	-0.102	0.960 ^{**}
PDQ (mm)	-0.395	-0.557	0.181
PC1	-0.285	-0.295	0.867 ^{**}
PC2	0.033	-0.377	-0.244

** and ^m indicate Pearson correlations between traits and environmental variables were significant ($P < 0.01$) and marginal significant ($0.05 < P < 0.1$), respectively.

1 to group 2 while Pn increased (Fig. 4, a). Minimum temperature of the coldest month (MTCM) was colder and temperature annual range (TAR) was larger in group 1 than in group 2 (Fig. 4, b).

Due to the fact that similar species are distantly related for convergent traits, we tested the correlation between these traits and environmental variables. P_{50} exhibited a significant positive correlation with MAP, PWM and PWQ (Table 5), a marginal significant positive correlation with MAT and TAR and a negative correlation with MTCM. Marginal significant correlation was also found between LSD and MAT. Considering the fact that a single variable may be inadequate to explain the phylogeny, principal component analysis (PCA) was used to examine all environmental variables. Consistent with the relationship found between traits and single environmental variables, there was no significant correlation between LSD, $\delta^{13}\text{C}$ and PCA scores and there was a significant positive correlation between P_{50} and the PC1 score (Table 5, see Tables S2 and S3 in the Supplementary Data).

Discussion

Ecological divergences between sister lineages

We considered eight environmental variables for eight phylogenetic nodes (Fig. 2, Table 2), and then analyzed minimum average temperature of the coldest month (Fig. 3), because low temperature is the main factor that can restrict the growth and distribution of alpine trees (Alvarez-Uria & Koerner, 2007; Manel et al., 2012). There is no doubt that temperature shown strongly divergent for basal nodes in the phylogeny (MTWM and TAR at node 1, MTCM and TAR at node 2 and 5) and precipitation is strongly

divergent for the terminal nodes. Therefore, based on the principle of seeva, the result suggests that ancestors of the current taxa grew in different environment during the development of *Picea* phylogeny. Slight divergence in sect. *Casicta* Mayr (*P. linkiangnesis* var. *likiangensis*, *P. linkiangnesis* var. *rubescens*, *P. linkiangnesis* var. *linzhiensis*) and *P. spinulosa* may be due to their recent differentiation (Lockwood et al., 2013) and similar distribution in low latitudes (Fig. 1, Table 1). We referred to the Lockwood et al. (2013) results and found that these *Picea* species diverged mostly during the Miocene epoch and partly at the Pliocene. Coincidentally, the QTP uplift during this time caused the east of China to become a south-east monsoon region, while the north-west turned into an arid inland region (Zhang et al., 2000). In more detail, the divergence time of node 1, 5, 6, and 7 was in the period between the end of the second uplift and the beginning of the third uplift of Qinghai-Tibet Plateau (QTP) (Shi et al., 1998). But QTP was not high enough to keep water vapor from going deep into the East Asia continent, which would create favorable conditions for extensive forest expansion (for ancestors) (Shi et al., 1998). Due to the uplift of different regions and degrees of QTP during this long geological period, it triggered the divergence of *Picea* species. Since then, the distributional ranges of species of these nodes may have been affected and finally located in the southeastern and southern edge of the QTP, where the climate was warmer and wetter than the northwest (Li et al., 2013; Sun et al., 2015; Feng et al., 2018). The divergence time of node 2, 3 and 8 was in the Quaternary period (Li et al., 2013; Bi et al., 2016). At this time, the QTP rose rapidly near to its present height, but climate showed an apparent pattern of alternating glacial and interglacial periods (Shi et al., 1998). Glacial and interglacial affected the biotic communities moved north and south through migration of climatic zones (Liu et al., 2014), which has been basically consistent with the appearance of modern plants. In addition, the result for the variable 'minimum average temperature of the coldest month' was also associated with significant divergence for the basal nodes. Hence, we speculate that this pattern of *Picea* divergence may mirror the contemporaneous ecological split in China, but the real situation needs more analysis of evolutionary history and biogeography.

Relationship between conservative/convergent traits and environmental variables

We used QVI to quantify levels of conservative/convergent evolution for each trait. Our results

suggested that LMA and Pn are conservative traits while LSD, $\delta^{13}\text{C}$ and P_{50} are convergent traits. Conservative traits here were defined as closely related species that are phenotypically similar (Ackerly & Reich, 1999). According to the results of seeva and Fig. 4, group 1 tended to occupy a colder and larger TAR environment than group 2 while LMA was higher and Pn was lower for group 1 than group 2. These results indicate that the variation trends with respect to conservative traits were retained preferentially by ancestors during the separation of environmental variables (Ackerly & Donoghue, 1998). Because high LMA (thick needles and large osmotic adjustments) are beneficial to species survival in cold environments, Pn is influenced indirectly (Coble & Cavaleri, 2014).

For convergent traits, only P_{50} showed significant positive correlations with MAP, PWM and PWQ. Generally, species with more negative P_{50} tend to occupy more arid habitats, which has been regarded as being because increased cavitation resistance (low P_{50}) is a key attribute of species growing in drought-prone habitats (Pockman & Sperry, 2000; Cochard et al., 2008). Moreover, the relationships between P_{50} and temperature (MAT, MTCM and TAR) suggested P_{50} also played a role in freezing tolerance (Cobb et al., 2007). However, neither LSD nor $\delta^{13}\text{C}$ showed significant relationships with the eight environmental variables even when further analyzed on the basis of PCA scores. This suggests that these variables were not the main factors which led to convergence.

In summary, our analysis revealed that the nine *Picea* species exhibit a distinct environmental divergence pattern: strong divergences at the basal nodes were associated with temperature variables, whilst the divergences at the terminal nodes were associated with precipitation variables. Comparative results of functional traits in the common garden indicated that differentiation of traits reflects genetic differences (inherent differences or adaptation) between species. Further analysis suggested LMA and Pn were conservative traits while LSD, $\delta^{13}\text{C}$ and P_{50} were convergent traits. The comprehensive results illustrated that LMA and Pn were selectively retained when their ancestors were subjected to cold environmental separation. P_{50} was an important trait with respect to adaptation to precipitation differences. Although we explored the environmental divergence and trait differentiation among *Picea* species through seeva and QVI, a more complete understanding of the relationship between environment and functional traits during species evolution still needs further research. For example, mutual verification of environmental divergence and real environment change is required by examining more species and traits to find the key trait at different stages or during the whole history of the taxa.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (Grant Nos. 31522013, 31370603 and 31170571) and the Fundamental Research Funds for the Central Universities (lzujbky-2016-ct10).

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