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Non-linear response of stomata in *Pinus koraiensis* to tree age and elevation

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Abstract Knowledge on variations in stomata is useful in reflecting leaf physiological characteristics of CO_2 uptake and water transpiration, and predicting the responses of plants to future climate change. Stomatal density and number of stomatal rows (current-year, 1- and 2-year-old needles) in relation to tree age (ranging from 25 to 320 years old), elevation (ranging from 738 to 1,380 m a.s.l.), and sun exposure (sun and shade exposure) were investigated in *Pinus koraiensis* trees. Stomatal density and number of stomatal rows in relation to tree age and elevation showed a humped curve with the maximum values at intermediate levels of tree age (210 years old) and elevation (1,050 m a.s.l.), respectively. Needle age but not sun exposure significantly affected the stomatal density across tree ages and elevations. Our results suggest that variations in stomatal density of *Pinus koraiensis* needles are related to ontogenetic growth and environmental factors.

Keywords Altitude · Needle age · Stomatal density · Stomatal row

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Introduction

Stomata play an important role in regulating CO_2 uptake for photosynthesis and water loss for transpiration (Al Afas et al. 2006; Casson and Gray 2008). Thus, assessing stomatal number of trees is critical to understand gas exchange between forests and atmosphere, in particular when considering future climate change scenarios. Stomatal density (the number of stomata per unit leaf area) is highly species specific, ranging from 5 to 1,000 mm^{-2} (Holland and Richardson 2009), and varies with growth environments, such as CO_2 concentrations (Lin et al. 2001; Soares et al. 2008), temperature (Luomala et al. 2005), humidity (Serna and Fenoll 1997) and light intensity (Casson and Gray 2008). The initiation and distribution of needle stomata differ from those of stomata on leaves. Needle stomata occur at the base of the needle and develop in longitudinal files during needle growth (Croxdale 2000). Thus, stomatal row is also an important parameter for assessment of stomatal number.

Generally, locations at higher elevation are characterized by lower temperature, lower CO_2 partial pressure but higher UV irradiance. The natural gradients of environmental factors along elevations may reflect the confounding effects of environments on stomatal behaviors. It has been found that plants grown in cooler climates have in general more stomata than in warmer climates (Körner and

Larcher 1988). *Pinus sylvestris* needles grown at elevated temperature (+2.8 to +6.2 °C) showed lower stomatal density than those grown at ambient temperature (Luomala et al. 2005). Based on the studies of broad-leaved trees, shade leaves have lower stomatal density than sun leaves (Al Afas et al. 2007; Loranger and Shipley 2010). Since altering irradiance can induce variation in guard cell length (Lomax et al. 2009), stomatal density changes with light intensity (Beaulieu et al. 2008). Based on data from fossilized or herbarium leaves, it is known that stomatal density decreased with increasing atmospheric CO_2 concentration (Woodward 1987; Kouwenberg et al. 2003). Since temperature and CO_2 partial pressure consistently decrease with elevation (Kouwenberg et al. 2007), stomatal density is expected to increase along elevations based on the documents mentioned above. The positive relationship between stomatal density and elevation has been reported for some species (e.g. Woodward et al. 2002; Kouwenberg et al. 2007). However, the relationship is not always presented in all species. For example, stomatal density of *Picea crassifolia* needles increased with elevation when elevation was below 3,000 m a.s.l., but decreased with elevation when elevation was above 3,000 m a.s.l. (Qiang et al. 2003). Körner et al. (1986) reported that the changes in stomatal density with elevation were strongly related to light and life form of plants. For example, *Nothofagus* and *Griselinia* showed no significant elevational changes in stomatal density while ericaceous shrubs and herbaceous *Ranunculus* showed a significant increase in stomatal number with elevation (Körner et al. 1986).

A natural forest is dominated by coniferous or/and broad-leaved trees with different ages. Leaf morphology, anatomy, physiology, chemistry differed significantly among different aged trees, suggesting a developmental change in needle anatomy and morphology with increasing tree age (Day et al. 2001; Apple et al. 2002; Niinemets 1997, 2002; England and Attiwill 2006). Leaf size and specific leaf area of *Eucalyptus regnans* decreased with increasing tree age, but stomatal frequency did not show clear trends with tree age despite of significant differences among age classes (England and Attiwill 2006). The number of stomatal rows on a needle generally does not change during ontogeny, but stomatal density may change with increasing leaf age due to the changes in cell expansion and number of epidermal cells during leaf development (Beerling and Royer 2002; Kouwenberg et al. 2004). Ferris et al. (1996) found that stomatal density of *Lolium perenne* leaves changed with leaf development, showing lower stomatal density in spring when leaves were younger and higher stomatal density in summer when leaves were mature. However, the stomatal density of young leaves in *Populus* spp. was larger than that of mature leaves (Ceulemans et al. 1995). Kouwenberg et al. (2004) reported that the increase in needle width during leaf maturation was the result of lateral expansion of the stomatal and epidermal cells, indicating that no extra stomatal rows or stomata occurred. Thus, data on changes in stomatal density or stomatal row with tree and leaf age to date are still insufficient. It seems necessary to study stomatal responses to tree and leaf age since the way in which stomata control photosynthesis and transpiration is a key determinant of plant growth and water status (Morison 2001).

We investigated age-related trends in needle stomata for juvenile, mid-age, and old *Pinus koraiensis* trees. Stomatal density (No_{stom} mm^{-2}), number of stomatal rows, distances between stomatal rows and between stomata within rows for different needle age classes (current-year and 1-year-old needles) at two canopy positions (sun and shade exposure) of *P. koraiensis* trees were measured. Trees with 5 tree age classes (mean ages 25, 120, 210, 260 and 320 years) were sampled at 738 m a.s.l. In addition, responses of stomatal density, number of stomatal rows, distances between stomatal rows and between stomata within rows of *P. koraiensis* to elevation were also investigated. *P. koraiensis* trees with the same age (mean 150 years old) were sampled along an elevational gradient (738, 1,050, 1,200, 1,380 m a.s.l.) of Changbai Mountain. Needle samples including three needle age classes (current-year, 1- and 2-year-old needles) at two canopy positions (sun and shade exposure) were selected. We hypothesize that (1) stomatal density and number of stomatal rows differ among tree and needle age classes; (2) stomatal density and number of stomatal rows increase with elevation; (3) there are significant differences in stomatal density and number of stomatal rows between sun exposed and shaded canopy positions.

Materials and methods

Field sites and materials

Changbai mountain (summit elevation of 2,734 m a.s.l.), a dormant volcano, is the highest mountain in northeastern China (42°24′N, 128°05′E). The first experimental site was located within the conifer/broadleaved mixed forest zone at 738 m a.s.l., where the annual mean air temperature is 3.6 °C and the mean annual precipitation is 695 mm. The highest mean monthly air temperature is about 20 °C, occurring during July and August; and the lowest temperature is below −20 °C, occurring in January. *P. koraiensis* with five tree age classes (mean ages 25-, 120-, 210-, 260- and 320-year) were selected for this study. The mean diameters at breast height were 2.5 ± 0.4, 25.6 ± 2.3, 43.0 ± 1.3, 57.0 ± 0.8 and 70.7 ± 2.5 cm for each tree age class, respectively. Tree age was calculated by growth process table of *P. koraiensis* established by Local Forestry Bureau in 1973. The growth process table of *P. koraiensis* showed the relationship between tree age and diameter at breast height. We estimated tree age of *P. koraiensis* at different elevations according to the diameters at breast height. Needle longevity of *P. koraiensis* is 2 years (current-year and 1-year-old needles) at elevation of 738 m a.s.l. The second experimental site with four elevations (738, 1,050, 1,230 and 1,380 m a.s.l.) was selected on the north-facing slope of Changbai Mountain. The mean diameters at breast height were 25.6 ± 2.3, 30.7 ± 1.5, 34.0 ± 4.0 and 34.5 ± 5.0 cm for each elevation, respectively. Above 1,400 m a.s.l., no *P. koraiensis* trees were found. 2-year-old needles still survive at elevations of 1,050, 1,200 and 1,380 m a.s.l. except for the elevation of 738 m a.s.l. Current-year, 1- and 2-year-old needles (no 2-year-old needles at 738 m a.s.l.) on sun and shade exposed upper canopy levels of trees at the 4 elevations were sampled to analyze stomatal density and number of stomatal rows. Three trees were sampled and analyzed for each tree age class at each elevation.

Stomata counts

Twenty fresh current-year, 1- and 2-year-old needles were, respectively, sampled from sun and shade exposed branches within the upper canopy. The middle portions of fresh needles of 1 cm length were cut for stomatal counting. Needles of *P. koraiensis* have three surfaces, but only two surfaces have stomata. No significant differences in stomatal density and number of stomatal rows were found between the two surfaces (data not shown). Therefore, only one surface was used to count stomata. The epidermis was torn apart for counting the number of stomatal rows on the needle surface, measuring the distance between stomatal rows and between stomata under 10 × 20 magnification with Nikon Eclipse 80i microscope. At least five fields of view on each slide were randomly selected for stomatal counting. Stomatal density was expressed as the number of stomata per square millimeter of needle area.

Statistical analysis

All data were tested for normality and homogeneity of the variances prior to statistical analyses. Three-way ANOVAs were applied to test the effects of tree age, canopy position (sun and shade exposure), and needle age (current-year and 1-year-old) on stomatal density, number of stomatal rows, mean distance between stomata, and mean distance between stomatal rows at the same elevation (738 m a.s.l.). One-way ANOVA was used to evaluate the response of stomatal parameters to tree age within each needle age class when canopy position was not taken into consideration. Post hoc comparison tests were used if the differences among tree age classes were significant. Three-way ANOVAs were applied to test the effects of elevation, canopy position (sun and shade exposure) and needle age (current-year, 1-year-old and 2-year-old) on the four stomatal parameters. One-way ANOVA was used to evaluate the responses of stomatal parameters to elevation within each needle age class when canopy position was not taken into consideration. Post hoc comparison tests were used if the differences among elevations were significant. All analyses were performed with SPSS 13.0 software (SPSS Inc. Chicago, IL, USA).

Results

Variation in stomatal parameters between sun and shade exposure

Number of stomatal rows, stomatal density, distances between stomatal rows and between stomata on sun exposed canopy positions did not significantly differ from the samples taken at shaded canopy positions among different aged trees and along elevation gradients (Tables 1, 2). The number of stomatal rows ranged from 3.6 to 6.9 and stomatal density ranged from 98.6 to 158.7 stomata mm^{-2} within 5 tree age classes at the elevation of 738 m a.s.l. (Table 3). The maximum and minimum numbers of stomatal rows were 5.8 and 4.4, and maximum and minimum stomatal density were 105.2 and 131.5 stomata mm^{-2} across needle age classes within 4 elevation gradients (Table 4).

Variation in stomatal parameters among tree ages

Number of stomatal rows, stomatal density, distances between stomatal rows and between stomata differed

Table 1 Effects of tree age, canopy position and needle age on stomatal parameters of *Pinus koraiensis*, tested with three-way ANOVAs

	SR	SD	DSR	DS
Tree age (Ta)	**	**	**	**
Canopy position (P)	ns	ns	ns	ns
Needle age (Na)	ns	**	ns	**
Ta × P	**	ns	*	ns
Ta × Na	ns	*	ns	*
P × Na	ns	ns	ns	*
Ta × P × Na	ns	**	ns	ns

SR number of stomatal rows, *SD* stomatal density, *DSR* distance between stomatal rows, *DS* distance between stomata

Trees were sampled at 738 m a.s.l. Levels of significance are indicated as: ** $P < 0.01$, * $P < 0.05$ and ^{ns} $P \geq 0.05$

Table 2 Effects of elevation, canopy position and needle age on stomatal parameters of *Pinus koraiensis*, tested with three-way ANOVAs

	SR	SD	DSR	DS
Elevation (E)	**	**	**	**
Canopy position (P)	ns	ns	ns	ns
Needle age (Na)	**	**	**	ns
E × P	ns	ns	ns	ns
E × Na	ns	ns	*	ns
P × Na	*	ns	ns	ns
E × P × Na	ns	*	ns	ns

SR number of stomatal rows, *SD* stomatal density, *DSR* distance between stomatal rows, *DS* distance between stomata

Trees were investigated with similar ages (approx. 120–150 years old). Levels of significance are indicated as: ** $P < 0.01$, * $P < 0.05$ and ^{ns} $P \geq 0.05$

significantly among tree age classes at the elevation of 738 m a.s.l. (Table 1). Since no significant differences in stomatal parameters were found between canopy positions, we pooled data from sun and shade exposed needle samples.

Within the chronosequence of 25-, 120-, 210-, 260- and 320-year-old trees, needles from the 210-year-old trees had the greatest and from the 25-year-old trees had the lowest number of stomatal rows and stomatal density compared to the other age classes (Table 3). No significant differences in the number of stomatal rows and stomatal density were observed among 120-, 260- and 320-year-old trees (Table 3). The average number of stomatal rows from 120-, 260- and 320-year-old trees at lower elevation was 25.1 % lower than that from 210-year-old trees and 24.8 % higher than that from 25-year-old trees for current-year needles; and 22.1 % lower than that from 210-year-old trees and 27.2 % higher than that from 25-year-old trees for 1-year-old needle. The stomatal density of current-year needles from 210-year-old trees was 35.5, 22.9, 28.9 and 19.5 % higher than that from 25, 120, 260 and 320-year-old trees, respectively. For 1-year-old needles, stomatal density from 210-year-old trees was 26.0, 15.9, 26.9 and 16.3 % higher than that from 25, 120, 260 and 320-year-old trees (Table 3). The higher stomatal density of 210-year-old trees was mainly attributed to the significant decrease in the distances between stomatal rows and between stomata (Table 3).

Despite of significant differences in the number of stomatal rows and stomatal density among age classes, there was no linear pattern between number of stomatal rows, stomatal density and tree age. There was a watershed in 210-year-old trees, indicating that number of stomatal rows and stomatal density increased before trees reach this age and decreased thereafter.

Table 3 The number of stomatal rows (No.), stomatal density (No. mm^{-2}), distances between stomatal rows and between stomata (μm) of *Pinus koraiensis* needles at each tree age class

	At 25 years		At 120 years		At 210 years		At 260 years		At 320 years	
	Sun	Shade	Sun	Shade	Sun	Shade	Sun	Shade	Sun	Shade
Current-year needles										
SR	3.8 ± 0.2	4.0 ± 0.1	5.0 ± 0.1	4.9 ± 0.2	6.9 ± 0.2	6.0 ± 0.2	4.6 ± 0.2	4.8 ± 0.2	5.0 ± 0.2	4.7 ± 0.2
SD	99.1 ± 3.7	100.7 ± 2.5	124.5 ± 5.4	114.0 ± 3.5	150.8 ± 5.4	158.7 ± 5.6	116.3 ± 5.0	103.6 ± 3.7	120.1 ± 3.2	129.0 ± 4.5
DSR	139.0 ± 4.9	127.4 ± 2.6	117.7 ± 3.1	124.9 ± 3.7	100.8 ± 2.9	96.0 ± 2.4	122.6 ± 4.3	130.0 ± 4.9	119.2 ± 2.9	117.4 ± 3.3
DS	78.4 ± 1.6	80.5 ± 2.0	73.6 ± 1.5	75.8 ± 1.4	67.3 ± 1.4	69.6 ± 1.5	76.6 ± 1.4	80.1 ± 1.4	73.0 ± 1.1	71.5 ± 1.5
1-year-old needles										
SR	3.6 ± 0.1	4.0 ± 0.1	4.9 ± 0.1	4.9 ± 0.1	6.3 ± 0.2	5.5 ± 0.2	4.6 ± 0.2	4.7 ± 0.2	5.0 ± 0.3	4.7 ± 0.2
SD	98.6 ± 3.0	109.0 ± 2.9	111.7 ± 2.5	124.2 ± 6.7	148.0 ± 5.9	132.5 ± 3.9	104.2 ± 3.6	100.8 ± 3.5	116.4 ± 5.0	118.4 ± 3.3
DSR	134.1 ± 4.9	123.1 ± 3.3	122.5 ± 2.7	119.2 ± 4.0	100.0 ± 2.9	108.4 ± 2.5	128.1 ± 4.4	127.1 ± 3.8	123.6 ± 4.4	120.1 ± 3.3
DS	80.0 ± 1.5	76.9 ± 1.3	77.8 ± 1.4	76.3 ± 1.3	71.8 ± 1.2	68.8 ± 1.6	82.1 ± 1.8	85.1 ± 1.8	72.8 ± 1.1	72.3 ± 1.1

Trees were sampled at 738 m a.s.l. Mean ± SE

SR number of stomatal rows, *SD* stomatal density, *DSR* distance between stomatal rows, *DS* distance between stomata

Table 4 The number of stomatal row (No.), stomatal density (No.mm^{-2}), distances between stomatal rows and between stomata (μm) of *Pinus koraiensis* needles at each elevation

	738 m a.s.l.		1,050 m a.s.l.		1,200 m a.s.l.		1,380 m a.s.l.	
	Sun	Shade	Sun	Shade	Sun	Shade	Sun	Shade
Current-year needles								
SR	5.0 ± 0.1	4.9 ± 0.2	5.5 ± 0.3	5.2 ± 0.2	5.2 ± 0.3	4.4 ± 0.1	5.2 ± 0.2	4.9 ± 0.2
SD	124.5 ± 5.4	114.0 ± 3.5	118.5 ± 3.9	120.7 ± 4.4	105.9 ± 3.2	105.2 ± 3.3	115.8 ± 2.6	118.4 ± 3.0
DSR	117.7 ± 3.1	124.9 ± 3.7	118.7 ± 3.7	114.4 ± 3.4	124.7 ± 4.3	127.9 ± 4.0	113.6 ± 2.7	117.1 ± 2.8
DS	73.6 ± 1.5	75.8 ± 1.4	75.0 ± 1.2	75.8 ± 1.1	80.1 ± 1.5	77.6 ± 1.7	78.7 ± 1.5	76.9 ± 1.2
1-year-old needles								
SR	4.9 ± 0.1	4.9 ± 0.1	5.8 ± 0.3	5.3 ± 0.3	5.0 ± 0.1	5.2 ± 0.2	5.5 ± 0.5	5.1 ± 0.2
SD	111.7 ± 2.5	124.2 ± 6.7	124.9 ± 2.8	121.9 ± 3.7	114.8 ± 3.7	121.5 ± 3.8	122.3 ± 3.4	116.8 ± 2.3
DSR	122.5 ± 2.7	119.2 ± 4.0	111.9 ± 2.1	113.4 ± 2.6	115.3 ± 2.0	111.6 ± 2.7	112.4 ± 2.9	111.4 ± 2.6
DS	77.8 ± 1.4	76.3 ± 1.3	74.3 ± 1.2	75.0 ± 0.7	77.7 ± 1.4	78.5 ± 1.5	76.6 ± 1.3	80.3 ± 1.1
2-year-old needles								
SR	–	–	4.8 ± 0.2	4.9 ± 0.1	4.8 ± 0.1	4.9 ± 0.1	4.6 ± 0.2	4.8 ± 0.2
SD	–	–	126.6 ± 4.2	131.5 ± 3.9	117.6 ± 3.5	121.2 ± 3.5	116.7 ± 3.0	122.8 ± 3.7
DSR	–	–	108.7 ± 3.8	103.4 ± 2.4	116.4 ± 3.5	112.5 ± 3.0	113.9 ± 2.5	114.5 ± 4.3
DS	–	–	76.7 ± 1.3	73.7 ± 1.5	76.3 ± 1.1	75.4 ± 0.9	78.7 ± 1.3	75.8 ± 1.1

Trees were investigated with similar ages (approx. 120–150 years old). Mean ± SE

SR number of stomatal rows, *SD* stomatal density, *DSR* distance between stomatal rows, *DS* distance between stomata

"–" indicated that no 2-year-old needles survived at the elevation of 738 m a.s.l

Variation in stomatal parameters among elevations

Number of stomatal rows, stomatal density, distances between stomatal rows and between stomata were observed to be significantly affected by elevation (Table 2). Since no significant differences in stomatal parameters were found between sun and shade needles, we pooled data from both canopy positions. For each needle age class, the variation in elevation effects was not always significant for stomatal parameters ($P > 0.05$). No clear correlation of stomatal parameters with elevation was observed, although the number of stomatal rows and stomatal density of needles from 1,050 m a.s.l. was relatively higher. The average number of stomatal rows was 5.0, 5.3, 4.9 and 5.0 at elevations of 738, 1,050, 1,200 and 1,380 m a.s.l. across three needle ages, respectively. Similarly, the average stomatal density was 118.5, 124.0, 114.3 and 118.8 stomata mm^{-2} at elevations of 738, 1,050, 1,200 and 1,380 m a.s.l, respectively.

Variation in stomatal parameters among needle age

At an elevation of 738 m a.s.l, needle age affected stomatal density but not number of stomatal rows among different tree age classes (Table 1). Current-year needles had a significantly higher stomatal density than 1-year-old needles except for 25-year-old trees when compared at the same tree age (Tables 1, 3). The relatively higher stomatal density in current-year needles was mainly due to the decrease in distance between stomata (Tables 1, 3). On average, stomatal density in current-year needles was 4.3 % higher than in 1-year-old needles across all tree ages. The greatest difference (10.3 %) in stomatal density was found between 1-year-old and current-year needles in 210-year-old trees.

Needle age significantly affected number of stomatal rows, stomatal density, and distance between stomatal rows, but not distance between stomata along elevation gradients (Table 2). 1-year-old needles had a relatively higher number of stomatal rows and 2-year-old needles showed higher stomatal density across four elevation gradients. The average number of stomatal rows in current-year, 1- and 2-year-old needles was 5.1, 5.2 and 4.8, respectively; and average stomatal density was 115.4, 120.0 and 122.7 stomata mm^{-2}, respectively.

Discussion

Effects of tree age on stomatal parameters

Trees show a variety of morphological, anatomical and photosynthetic changes as they grow up and age

(Niinemets 2002; England and Attiwill 2006; Boratyńska et al. 2008; Zhao et al. 2008). Consistent with our first hypothesis, tree age significantly affected stomatal parameters. We observed, as Boratyńska et al. (2008) did, that 25-year-old *P. koraiensis* trees had the lowest number of stomatal rows and stomatal density compared to the older trees. Since water transport and transpiration rate increase with tree age, stomatal density or stomatal number may increase with tree age (Apple et al. 2002; Zhao et al. 2008). Greenwood et al. (2008) also reported that there was a significant increase in stomatal density of red spruce (*Picea rubens* Sarg.) with increasing tree age. In our study, the number of stomatal rows and stomatal density of *P. koraiensis* needles increased with tree age only when tree age was less than 210 years, indicating that there was a transitional time period around 210-year-old trees of *P. koraiensis*.

It has widely been reported that photosynthetic and growth rate decrease with increasing age for most forest tree species (Yoder et al. 1994; Day et al. 2001; Niinemets 2002). This decline is accompanied by changes in foliar morphology and physiology (Greenwood et al. 2008). Photosynthetic CO_2 uptake and water loss via stomata are two antithetical processes, and stomatal density may develop towards an optimization of the ratio between CO_2 uptake and water loss during the evolution of plants (Raven 2002; Hetherington and Woodward 2003; Driscoll et al. 2006; Brodribb et al. 2009). The oldest *P. koraiensis* trees in northeastern China are approximate 400 years old. Therefore, we chose 210-year-old individuals as middle aged trees. After this age, photosynthesis and growth potential of *P. koraiensis* may begin to decrease. Ryan et al. (1997) also reported that growth and biomass accumulation tended to gradually decline after reaching a peak. Increased hydraulic resistance and decreased photosynthesis with tree age may be related to reducing stomatal density and number of stomatal rows in 260- and 320-year-old *P. koraiensis* trees.

Effects of needle age on stomatal parameters

We have found significant, needle age-related variations in stomatal density of *P. koraiensis*, supporting our hypothesis. Early theories indicate that stomatal density is established during an early stage of leaf development (Woodward 1987; Radoglou and Jarvis 1990). According to Lake et al. (2001), mature leaves can transmit external information to new leaves, inducing an appropriate adjustment of stomatal development. At the elevation of 738 m a.s.l., current-year needles of *P. koraiensis* had higher stomatal density than 1-year-old needles across tree ages. However, this does not imply that the absolute stomatal number decreases with needle development. The decrease in stomatal density in 1-year-old needles compared to current-year needles was mainly attributed to the increase in the distance between stomata, which may be due to epidermal cell expansion with needle growth and development.

On the other hand, stomatal density increased with needle age across elevations. The changes in total stomatal number were not as large as the changes in stomatal density due to the decrease in the number of stomatal rows with needle age (Table 4). Increased density and decreased number of stomatal rows with needle age indicate that needle width decreased over the course of the time. We measured needle length and width of three needle age classes (data not shown) and found that older needles were narrower and shorter than younger needles. Stomata do not disappear with needle development, but stomatal density may vary due to changes in epidermal cell number or expansion. Beerling and Royer (2002) found that stomatal density was quite susceptible to fluctuations in their growth environment, being directly related to leaf expansion. The higher number of stomatal rows in younger needles compared to older needles indicates that stomatal development is controlled by both inheritance information and environmental factors.

At the elevation of 738 m a.s.l. on the Changbai mountain, needles of *P. koraiensis* survive 2 years, whereas needles grown at elevations from 1,050 to 1,380 m a.s.l. survive 3 years. Plants grown at higher elevations are subjected to harsher environmental conditions during the processes of growth and development. If there is a high production of pinecones, the number of 2-year-old needles of *P. koraiensis* decreased sharply (data not shown). Therefore, needle life span seems to be closely associated not only with environmental conditions but also with the reproduction rate (Shaver 1981; Xiao 2003). The higher stomatal density in older needles and longer needle retention are adaption strategies to maintain growth and reproduction, while being exposed to harsher environmental conditions.

Effects of elevation on stomatal parameters

The present study showed that elevation significantly affected stomatal density of *P. koraiensis* needles. No clear elevation trend was found although number of stomatal rows and stomatal density of needles from 1,050 m a.s.l. was relatively higher, not supporting the second hypothesis. The elevation effects are a proxy of environmental factors of CO_2 partial pressure, air temperature, solar irradiance, precipitation and wind exposure. In theory, solar irradiance and precipitation gradually increase, but temperature and CO_2 partial pressure consistently decrease with elevation (Kouwenberg et al. 2007). The changes in these environmental factors along an elevation gradient could mutually counteract the individual parameter effects on stomata, resulting in an increase, decrease or no significant change in stomatal density.

Previous studies suggested that stomatal density increased with elevation in herbaceous plants, shrubs and perennial tree species (DeLucia and Berlyn 1984; Körner et al. 1986; Woodward et al. 2002; Kofidis et al. 2003; Kouwenber et al. 2007), but opposite results were also reported for apple trees, *Pinus flexilis*, *Abies lasiocarpa*, *Picea engelmannii* and *Pinus contorta* (Hultine and Marshall 2000; Schoettle and Rochelle 2000; Aslantaş and Karakurt 2009). Körner et al. (1986) observed that *Nothofagus menziesii* in the Southern Alps of New Zealand showed no change in stomatal density with elevation. Stomatal density of *Betula papayrifera* var. cordifolia and *Sorbus americana* also did not vary significantly with elevation on the Mountain Moosilauke of the USA. (Holland and Richardson 2009). In addition, variation in stomatal density with elevation for some species showed a transitional zone. The stomatal density of mature *Picea crassifolia* Kom. showed an initial increase at elevations between 2,501 and 3,060 m a.s.l., and then decreased at elevations above 3,060 m a.s.l. (Zhao et al. 2008). Similarly, Luo et al. (2006) also found that stomatal density of *Picea asperata* Mast. needles increased towards elevations below 2,950 m but decreased at elevations between 2,950 and 3,390 m a.s.l. Therefore, the responses of stomatal density to elevation may be not only species specific but also more complex than originally assumed. The responses of stomatal density or stomatal number to elevation for *P. koraiensis* needles are most likely to be related to counteract environmental factors of temperature, light, humidity and CO_2 concentration.

Effects of canopy position on stomatal parameters

Our findings of no significant differences in stomatal density and number of stomatal rows between shade and sun exposed needles do not support the third hypothesis. Previous studies indicated that stomatal characteristics were related to canopy position (England and Attiwill 2006). For example, Al Afas et al. (2007) reported that stomatal density was lower for shade leaves than for sun leaves because changing irradiance-induced changes in guard cell length and therefore in stomatal density (Beaulieu et al. 2008; Lomax et al. 2009; Loranger and Shipley 2010). The irradiance, air temperature and vapor pressure deficit in sun-exposed canopy leaves differ from those leaves grown in the shade canopy. Therefore, the apparent lack of responses of stomatal parameters to canopy position in our study may be a result of several counteracting environmental factors. Foliage can develop different mechanisms to accommodate the microclimate within the canopy (Oliveira et al. 1996; Li et al. 2001).

Conclusion

In the present study, stomatal density and number of stomatal rows of *P. koraiensis* differed among tree ages, among elevations and among needle ages, but not between canopy positions. The results of 210-year-old *P. koraiensis* with higher stomatal number suggest stomata may relate to tree growth potential because higher stomatal number may increases CO_2 exchange between trees and atmosphere. The response pattern of stomatal density to elevations indicates that stomata development is determined by genetic and environmental factors.

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