

# Germination and early seedling growth of *Pinus densata* Mast. provenances

Yulan Xu<sup>1,2</sup> · Nianhui Cai<sup>1</sup> · Bin He<sup>1</sup> · Ruili Zhang<sup>1</sup> · Wei Zhao<sup>3</sup> · Jianfeng Mao<sup>2</sup> · Anan Duan<sup>1</sup> · Yue Li<sup>2</sup> · Keith Woeste<sup>4</sup>

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**Abstract** We studied seed germination and early seedling growth of *Pinus densata* to explore the range of variability within the species and to inform afforestation practices. Phenotypes were evaluated at a forest tree nursery under conditions that support *Pinus yunnanensis*, one of the presumed parental species of *P. densata*. Seeds were collected from 20 open-pollinated trees within each of eight autochthonous populations representing the natural distribution of *P. densata* in China to assess variation in germination traits and early seedling growth, and to examine the relationships among these traits. Results showed that seeds from all populations germinated and seedlings established successfully. There were significant differences among populations in 13 of 14 traits evaluated. Seed

germination and early seedling growth were strongly related to seed size and seed weight. Bigger seeds germinated earlier and faster than small seeds, and seedling size was positively correlated with seed size. Some germination traits were strongly and significantly correlated with climatic variables associated with the provenance of the studied populations. Based on these observations, we conclude there were large, significant, and biologically important differences among *P. densata* populations in seed germination and seedling growth traits. The observed variability probably reflects a high degree of adaptive differentiation among populations that is likely to be relevant for future afforestation.

**Keywords** Afforestation · Conifer · Gaoshan pine · Provenance · Seed germination · Seed size · Seedling vigor

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✉ Yulan Xu  
xvyulan@163.com

- <sup>1</sup> Key Laboratory for Forest Resources Conservation and Use in the Southwest Mountains of China, College of Forestry, Southwest Forestry University, Kunming 650224, China
- <sup>2</sup> The State Engineering Laboratory for Tree Breeding, College of Biological Science and Biotechnology, Beijing Forestry University, Beijing 100083, China
- <sup>3</sup> State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Science, Beijing 100093, China
- <sup>4</sup> USDA Forest Service Hardwood Tree Improvement and Regeneration Center at Purdue University, West Lafayette, IN 47906, USA

## Introduction

*Pinus densata* Mast. (subgenus *Pinus*, a member of the hard pines, Diploxylon) or Gaoshan pine, is native to the Tibetan Plateau. It forms extensive forests at high elevations (Mao et al. 2009) in mountainous regions of southwestern and central China. *P. densata* is especially valued for afforestation and water conservation because it is shade intolerant, deep rooted, cold-resistant, drought-resistant, poor-site tolerant, and it may be deployed as a pioneer species on barren hillsides (Chinese Academy of Sciences 1978; Liu 1983).

The natural history of Gaoshan pine, especially its genetic history as a homoploid hybrid between *Pinus tabulaeformis* and *Pinus yunnanensis*, shapes the distribution of its phenotypic diversity (Gao et al. 2012). Harsh climatic conditions in the species' native range, which is

characterized by low temperature, high elevation, steep slopes, and frequent fires in inaccessible areas, have made afforestation by aerial seeding the most practical regeneration method for *P. densata* (Liu 1983). Under such conditions and especially where competition from shrubs or predation by rodents and birds is likely (Liu 1983; Yang 2005), fast germination and growth are essential for successful establishment. For this reason, these traits have been studied extensively in conifers, with larger seed size generally, but not always, associated with more rapid germination and seedling establishment (Gonzalez 1993; Parker et al. 2006; Wahid and Bounoua 2013).

The relationship of seed germination characteristics to seedling growth of *P. densata* have not been published, but high levels of provenance- and population-level variability are expected for these traits, as the species shows significant differences among populations in cone and seed morphometric traits (Mao et al. 2007). The evolutionary history of *P. densata* and its wide ecological range present an opportunity to investigate the association between the current habitats of *P. densata* and the germination and early seedling growth rate of seeds and seedlings. These factors are relevant to an understanding of the diversification of *P. densata* in response to landscape and climatic features, the proper management of *P. densata* genetic resources, and the deployment of these resources by forest nurseries. The association between height growth of pine provenances and their latitude and elevation of origin has been demonstrated (Lindgren et al. 1994). We evaluated seed germination and early seedling growth of *P. densata* from 20 open-pollinated trees within each of eight populations from three provenances. The goal of our research was to understand variation within and among *P. densata* populations for germination and early seedling growth traits, to examine the relationships among seed size and weight, germination, and seedling growth traits, and to associate observed variation in these traits with ecological and geographic variables.

## Materials and methods

### Study site

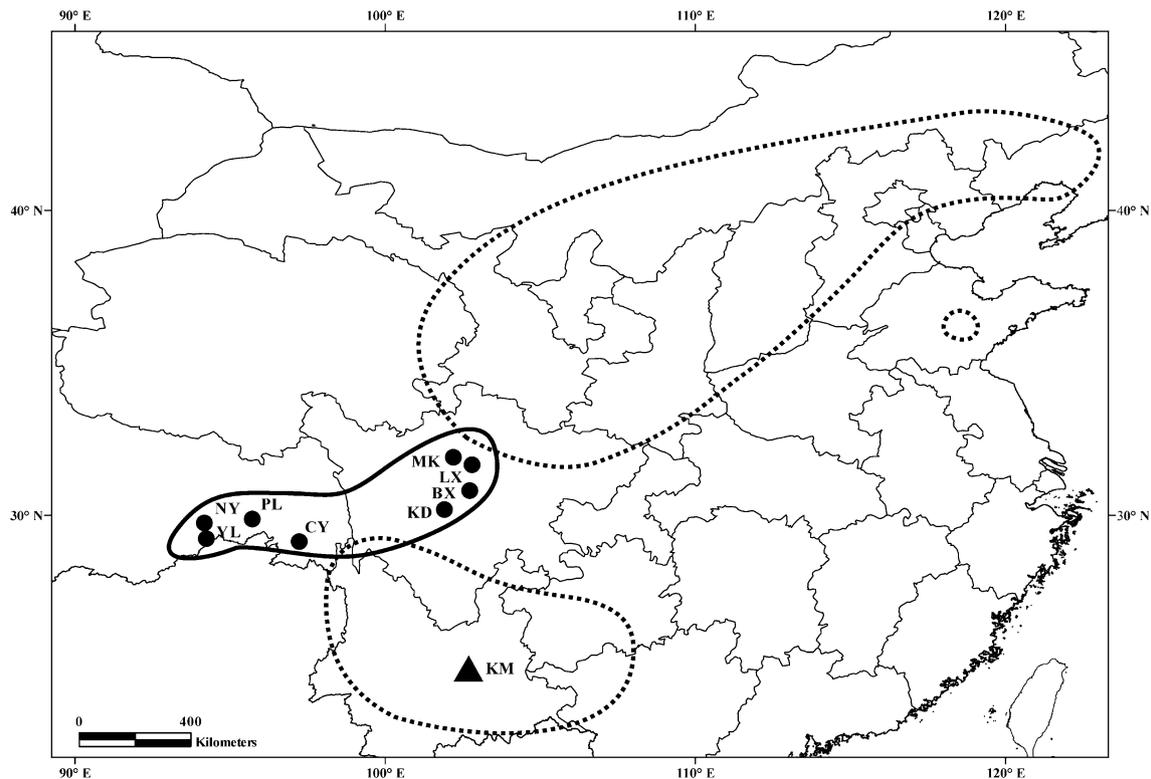
This study was conducted at the Forestry Department Nursery of Southwest Forestry University in Kunming, Yunnan, China (longitude: 102°45'41"E; latitude: 25°04'00"N; elevation: 1945 m; Fig. 1). The climate in Kunming is classified as low-latitude, mountain plateau monsoonal, with pronounced wet (June–October) and dry seasons. Annual precipitation is around 700–1100 mm, and the mean annual temperature is 14.7 °C (Wei et al. 2012). The mean air temperature at about 4:30 PM during the course of the experiment was 23.7 °C.

### Plant material

Sampled trees were within eight autochthonous populations representing central and marginal areas in the natural distribution of *P. densata* in China (Fig. 1; Table 1). The populations were selected based on previous investigations (Ma et al. 2006; Mao et al. 2009; Wang et al. 2011). Niyang (NY), Palung (PL), Yarlung (YL) and Cayu (CY) populations were located in the Plateau Temperate Zone, and others were in the Plateau Sub-temperate Zone. Seed germination and seedling growth were correlated with climatic data from the source provenances, including maximum and minimum temperature and precipitation in each month, and 19 other bioclimatic variables (BIO1–BIO19: annual mean temperature, mean diurnal range, isothermality, temperature seasonality, maximum temperature of warmest period, minimum temperature of coldest period, temperature annual range, mean temperature of wettest quarter, mean temperature of driest quarter, mean temperature of warmest quarter, mean temperature of coldest quarter, annual precipitation, precipitation of wettest period, precipitation of driest period, precipitation seasonality, precipitation of wettest quarter, precipitation of driest quarter, precipitation of warmest quarter, precipitation of coldest quarter) extracted from Worldclim (Global Climate Data, <http://www.worldclim.org/>; Hijmans et al. 2005) using each population's longitude and latitude. Each sampled tree was at least 100 m from any other sampled tree. Seeds used in this experiment were collected from 20 to 30 cones from the middle part of the crown of each of 20 healthy appearing, apparently even-aged (older than 35 years) open-pollinated trees. All cones were closed but fully mature. Cones were stored at room temperature until they dried and opened. Seeds were removed manually, their wings were removed, and the seeds were bulked and mixed by population. Some cones from every population did not open fully by ambient drying. These were soaked in warm water and dried in an oven at 40 °C for 24 h. The seeds extracted from each cone were floated in 100 % ethanol to separate filled from empty seeds (Mao et al. 2009), then stored at –20 °C until they were sown. Seed length (SL) and seed width (SW) were measured with an electronic vernier caliper, and thousand seed weight (TSW) was measured with a digital electronic balance. The ratio of length to width of seed (SLW) was calculated.

### Seed germination and experimental design

Empty or otherwise defective seeds were removed. Seeds were surface sterilized with 0.1 % HgCl<sub>2</sub> for 5 min, rinsed several times with distilled water, soaked in distilled water for 12 h and placed in seedbeds. Five replications of sixty



**Fig. 1** Distribution of *Pinus densata* populations evaluated in this study and of *P. tabuliformis* (northern China) and *P. yunnanensis* (southwestern China), the presumed parental species of *P. densata*.

The abbreviations are the same as in Table 1. The study site (Kunming) is indicated as KM

**Table 1** Location and collection details for eight populations of *Pinus densata* evaluated in this study

Population (Provenance) <sup>a</sup>	Region	Longitude (E)	Latitude (N)	Elevation (m)	Climate zone	Collection date
BX(I)	Baoxing, Sichuan	102°43'	30°45'	2330	Plateau subtemperate	Oct, 2008
CY(II)	Cayu, Xizang	97°13'	29°08'	3264	Plateau temperate	Nov, 2007
KD(II)	Kangding, Sichuan	101°55'	30°11'	2944	Plateau subtemperate	Oct, 2010
LX(I)	Lixian, Sichuan	102°48'	31°40'	2765	Plateau subtemperate	Oct, 2010
MK(I)	Maerkang, Sichuan	102°12'	31°55'	2709	Plateau subtemperate	Oct, 2010
NY(III)	Niyang valley, Xizang	94°10'	29°45'	3203	Plateau temperate	Nov, 2007
PL(II)	PalungZangbo, Xizang	95°42'	29°52'	2804	Plateau temperate	Nov, 2007
YL(III)	Yarlung Zangbo, Xizang	94°14'	29°14'	2960	Plateau temperate	Nov, 2007

<sup>a</sup> Provenances as described in Gao et al. (2012)

seeds were sown in a plot, which consisted of seeds from one population. The complete block experiment contained 40 plots ( $n = 2400$  seeds: 60 seeds  $\times$  8 plots  $\times$  5 replicates). All seeds were sown on a single day at a spacing of 5  $\times$  15 cm and were buried just below the soil surface (about 0.4–0.5 cm in depth) in April, 2011. The study site was irrigated using sprinklers to maintain soil moisture during germination. Seeds were considered to have germinated when the shoot emerged and exhibited normal growth and morphology (Tilki and Alptekin 2006). The

number of germinated seeds was counted every day, and time was recorded for calculating the initial (germination initial time, GIT) and final emerging shoot. Final germination time was defined as the day when no further germination occurred for four successive days. The interval from germination initiation to final germination was deemed germination duration (GD). Other traits including germination percentage (GP), mean daily germination (MDG), mean germination time (MGT), coefficient of germination (CG), germination index (GI), peak value

(PV), germination value (GV) and vigor index (VI) were calculated using the method of Tao and Zheng (1991), Czabator (1962), Akinci and Akinci (2010), Brown and Mayer (1988) and Bhattacharya et al. (2012). GV is an index combining speed and completeness of germination where larger values indicate faster and more complete germination (Czabator 1962). The traits were calculated as follows:

$$GP = \left( \frac{G}{T} \right) \times 100 \quad (1)$$

$$MDG = \frac{GP}{Gd} \quad (2)$$

$$MGT = \frac{[\sum (Gt \times Dt)]}{G} \quad (3)$$

$$CG = 100 \times \left[ \frac{G}{\sum Gt \times Dt} \right] \quad (4)$$

$$GI = \sum \left( \frac{Gt}{Dt} \right) \quad (5)$$

$$PV = \frac{Gpt}{Dpt} \quad (6)$$

$$GV = PV \times MDG \quad (7)$$

$$VI = SLL \times GP \quad (8)$$

where  $G$  is the total number of germinated seeds,  $T$  is the number of sowed seeds,  $Gd$  is the number of days to complete germination,  $Gt$  is the number (out of 60 seeds per block per population) of germinated seeds at day  $t$ ,  $Dt$  is the days  $t$  from the start of the experiment,  $Gpt$  is the cumulative number of seeds already germinated on the day for which the rate of germination first decrease,  $Dpt$  is the day previous to the day on which the rate of germination first decreases,  $SLL$  is the seedling length at the end of the germination period. In general, seed germination can be considered to take place in three stages, an initial stage (Stage I) when few seeds germinate, a short phase when most seeds germinate that slows to an asymptote (Stage II), and a final stage when the asymptote nears its maximum (Stage III) (Fig. 2).

Two months after the completion of germination, we measured each seedling's morphology and growth traits including cotyledon number (CN) and cotyledon length (CL). Seedlings height (H) and ground-line diameter (D) were measured after 3 months of growth from five randomly sampled plants in each plot.

### Statistical analysis

Each trait was characterized using mean values (M) for each population. Variance among and within populations was calculated using PROC Nested of SAS v.9.3 (SAS Institute, v9.3, Carey, NC, USA). PROC NLMIXED of SAS was used to model seed germination of each population  $\times$  replicate independently ( $n = 40$ ) with a version of

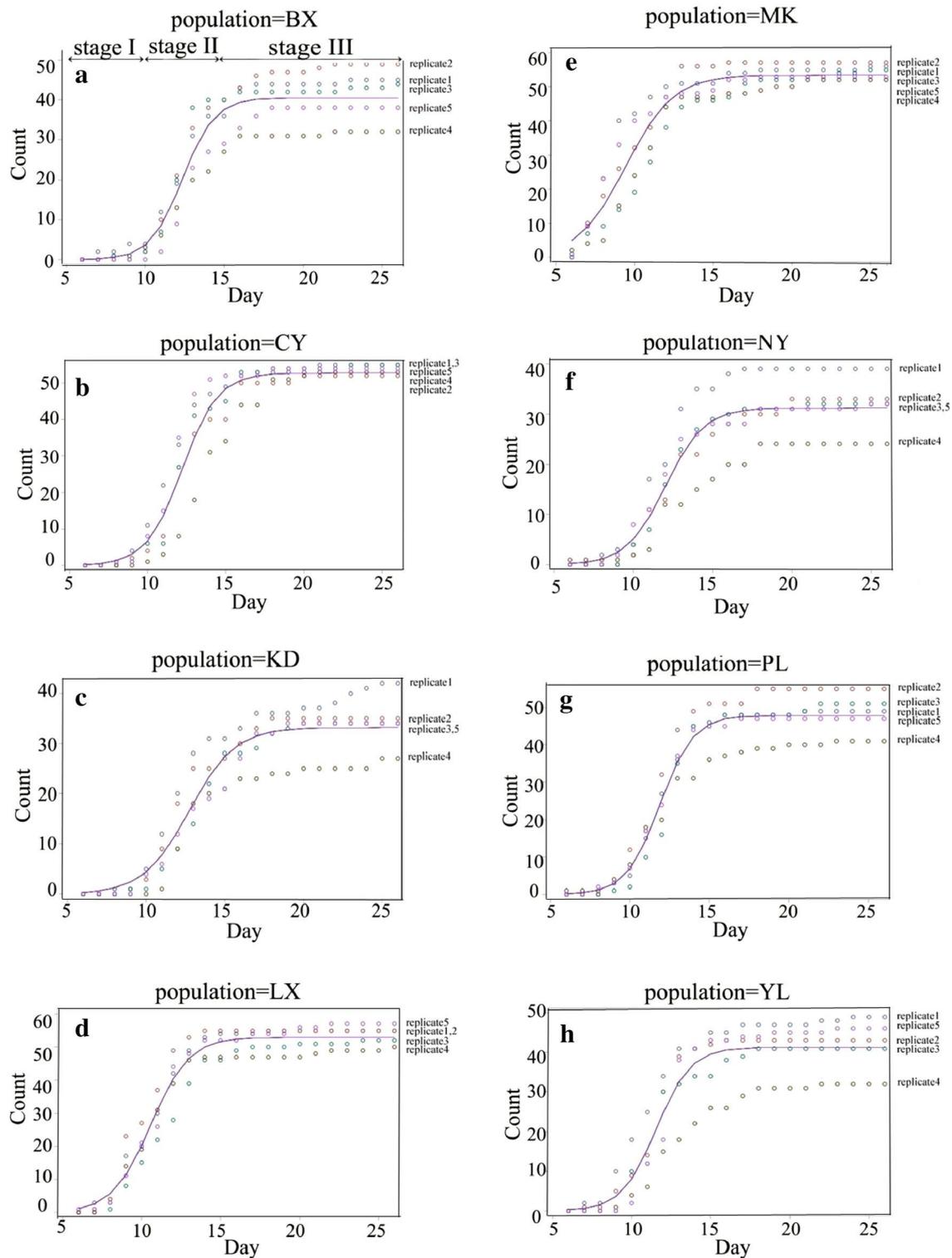
the logistic function (a generalized sigmoidal curve) widely used to model growth (Schimpf et al. 1977):

$$y = (b_1 + \mu_1) / 1 + \text{EXP}(-(day - b_2) / b_3) \quad (9)$$

We began iterations with  $b_1 = 60$ ,  $b_2 = 30$  and  $b_3 = 10$ , where  $(b_1 + \mu_1)$  represents the number of seeds that eventually germinated ( $b_1$  is an adjustment for each population where the overall mean is  $\mu_1$ );  $b_2$  is a constant of integration, and  $b_3$  is a germination rate per day. Final, optimized values for  $b_1$ ,  $b_2$ , and  $b_3$  for the best-fit line for each population were calculated using SAS PROC NLMIXED. Inflection points for the transitions from stages I to II (onset of rapid germination) and stages II to III (end of rapid germination) were determined for each population by taking the second derivative of the logistic curve and solving. The generalized logistic curve can be expressed as

$$y = \frac{k}{1 + e^{a-bt}}$$

The two inflection points are calculated as  $t = \frac{a \mp \ln(2 + \sqrt{3})}{b}$  (He et al. 2008). For NLMIXED, populations were fixed effects and replication (=block) was a random effect. The analysis of variance for count of seeds germinated each day was performed using SAS PROC GLIMMIX, multiple means tests were performed using Tukey's test. Provenance effects for germination counts were tested in a univariate model in GLIMMIX. When provenances were included in the linear model they were considered fixed effects and populations were nested in provenance for analysis. Seeds were pooled within populations, and TSW of each population was used as a covariate for analysis of provenance effects on seed germination. Seeds of provenance I (=group I of Gao et al. 2012) averaged 32.30 g, considerably larger than average (~20 g for all eight populations) and much larger than those of provenances II (12.84 g) and III (12.37 g) (provenances II and III correspond to groups II and III of Gao et al. 2012). Preliminary analysis using Levene's test showed that TSW was highly heteroscedastic. To improve the usefulness of TSW as a covariate when analyzing provenance effects, we log transformed the mean-centered TSW. Despite these adjustments, the relationship between log (TSW) and (predicted) GP remained non-uniform. Because TSW\*provenance had a highly significant effect ( $P = 0.0026$ ) on percent germination, we compared provenances for predicted germination percent at four key TSWs, i.e., the overall mean TSW and the mean TSW for each provenance. As previously mentioned, differences among provenances and populations for mean predicted germination were estimated separately for each day (e.g., day 9, 14, and 26, corresponding to stages I, II, and III, respectively) although for some days models failed to converge, especially when TSW was used as a covariate. So in all cases when least squares means (LSMEANS) of populations were generated, TSW was not included as a



**Fig. 2** Seed germination rate of eight *Pinus densata* populations (a–h). Data from each replicate is shown by *open circles*, and the best-fit line was added. The abbreviations are the same as in Table 1. In *panel*

a, division between germination stages as described in “[Materials and Methods](#)” section is shown

covariate. Analysis of covariance and differences among provenance and population means were performed using PROC GLIMMIX and Tukey’s test ( $P < 0.05$ ). In all cases

Kenward–Rogers adjusted denominator degrees of freedom were used. Data given in percentages were transformed by arcsine square root before ANOVA but the results are

**Table 2** Differences among provenances for seed germination traits

Provenance <sup>a</sup>	SL (mm)	SW (mm)	SLW	TSW (g)	GP (%)	MDG (%)	PV	MGT (d)	GV
I	6.9 ± 0.1a	4.0 ± 0.1a	1.75 ± 0.03a	32.30a	83.33 ± 3.02a	4.1 ± 0.3a	2.68 ± 0.17a	11.7 ± 0.2a	10.8 ± 0.8a
II	5.4 ± 0.1b	3.2 ± 0.1b	1.69 ± 0.03a	12.84b	76.00 ± 3.02b	3.7 ± 0.3a	2.05 ± 0.17b	13.2 ± 0.2b	8.0 ± 0.8b
III	5.4 ± 0.1b	3.2 ± 0.1b	1.70 ± 0.03a	12.37b	61.83 ± 3.16c	3.1 ± 0.3b	1.84 ± 0.20b	12.8 ± 0.2b	6.1 ± 0.9b
Means	5.95	3.49	1.71	20.02	75.21	3.69	2.23	12.54	8.57
Provenance	GI	GIT (d)	GD (d)	CG	VI	CN	CL (mm)	H (mm)	D (mm)
I	4.6 ± 0.2a	7.6 ± 0.3a	13.7 ± 1.1a	8.6 ± 0.2a	111.6 ± 10.3a	8.2 ± 0.2a	36.0 ± 2.3a	25.0 ± 1.0a	1.2 ± 0.1a
II	3.6 ± 0.2b	8.8 ± 0.3b	12.0 ± 1.1a	7.6 ± 0.2b	63.3 ± 10.3b	6.7 ± 0.2b	23.9 ± 2.3b	17.2 ± 1.0b	0.9 ± 0.1b
III	3.1 ± 0.2c	8.6 ± 0.4ab	11.81.2a	7.9 ± 0.2b	49.6 ± 10.5c	6.8 ± 0.2b	24.6 ± 2.4b	16.4 ± 1.1b	0.9 ± 0.1b
Means	3.84	8.30	12.58	8.06	77.96	7.30	28.61	19.94	1.04

Data are the Means ± 1 SD of 5 replications. Means followed by the same letter were not significantly different by Tukey's test ( $P > 0.05$ ). For ease of viewing, some tabulated data was rounded, but Tukey's ranks are shown as calculated with unrounded data

SL seed length, SW seed width, SLW seed length/seed width, TSW 1000-seed weight, GP germination percentage, MDG mean daily germination, PV peak value, MGT mean germination time, GV germination value, GI germination index, GIT germination initial time, GD germination duration, CG coefficient of germination, VI vigor index, CN cotyledon number, CL cotyledon length, H seedling height, D seedling ground-line diameter

<sup>a</sup> Provenances are genetic regions described in Gao et al. (2012)

reported as percentage. Spearman correlations were tested using PROC CORR of SAS.

## Results

### Seed size

Seeds of provenances and populations differed in size and shape. Seeds of Provenance I were both longer ( $P \leq 0.0001$ ) and wider ( $P \leq 0.0001$ ) than those of Provenances II and III, which did not differ in size from each other (Table 2). Seeds of populations Maerkang (MK) and Lixian (LX) were both significantly longer and wider than all other populations ( $P \leq 0.0001$ ). Seed shape (SL/SW) did not differ much, either by provenance or at the population level, although seeds of population MK were significantly more elongated than seeds of populations Kangding (KD) and YL. Differences in seed size were reflected in large differences in seed weight among provenances (TSW) (Tables 2, 3).

### Seed germination

In general, seeds began emerging in the first 5 days after planting. Emergence continued at a relatively low rate for an additional 5 days, at which point it increased rapidly. Over the next 3 days, about half the seeds germinated; then the seedling emergence rate slowed dramatically, but continued for another week or so. Thus, seed germination exhibited the expected sigmoidal curve over time (Fig. 2),

with some variation among replications (blocks). Each population\*replication was modeled using an exponential growth function, and in nearly every case (36 of 40 combinations) the model was highly significant ( $b_1$ ,  $b_2$ , and  $b_3$  values fit with  $P \leq 0.0001$ ) (Fig. 2). Over all populations, a mean of 58.2 % of seeds germinated during stage II, which lasted only 3.3 days on average (Fig. 2a). Average germination during stage I and stage III was 20.7 and 21.1 % and took place over 10.0 and 7.6 days, respectively. By 3 weeks after sowing, 75.2 % of seeds had germinated. There was no apparent relationship between length of time in storage and seed germination; populations collected in 2007 and 2010 showed both high and low GP (Tables 1, 3).

The fastest germination was recorded for seeds from MK and LX populations, with 8 and 9 days in stage I, respectively, but GIT did not differ significantly among populations (Fig. 2; Tables 1, 3). The MK, LX, and YL populations began stage II of germination by 3, 2, and 1 days, respectively, earlier than the remaining five populations (data not shown). Similarly, when we compared populations for germination on day 9, both provenance and population effects were significant ( $P = 0.0015$  and  $P = 0.003$ , respectively) (Tables 2, 4). Germination proceeded most rapidly during Stage II. Stage II corresponded to the period from day 10.7 to day 13.4 averaged across all 40 population × replication combinations, and there were no differences among populations for the amount of time required to complete this stage. On day 14, just after the end of Stage II, provenances and populations differed significantly for number of germinated seeds ( $P = 0.0004$  and 0.0006 respectively).

**Table 3** Means ± standard deviations for seed size, germination, and early seedling growth traits of *P. densata* populations

Populations <sup>a</sup>	SL (mm)	SW (mm)	SLW	TSW (g)	GP (%)	MDG (%)	PV	MGT (d)	GV
BX	5.4 ± 0.6b	3.2 ± 0.4b	1.7 ± 0.2 ab	16.2	69.3 ± 11.1bcd	3.2 ± 0.5ab	2.1 ± 0.7abc	13.3 ± 0.4c	6.9 ± 2.6bc
CY	5.3 ± 0.4b	3.1 ± 0.2b	1.7 ± 0.1ab	12.9	89.7 ± 2.2a	4.4 ± 0.4a	2.2 ± 0.5abc	13.1 ± 0.9c	9.6 ± 3.1ab
KD	5.2 ± 0.4b	3.1 ± 0.3b	1.7 ± 0.2 b	11.8	57.3 ± 8.9cd	2.7 ± 0.6b	1.4 ± 0.3bc	13.9 ± 0.5c	3.9 ± 1.1c
LX	7.4 ± 0.9a	4.4 ± 0.6a	1.7 ± 0.3 ab	39.2	89.7 ± 4.6a	4.6 ± 1.3a	2.8 ± 0.7a	11.4 ± 0.7ab	12.4 ± 3.6ab
MK	8.0 ± 1.6a	4.3 ± 0.7a	1.9 ± 0.3a	41.5	91.0 ± 3.5a	4.5 ± 1.0a	3.1 ± 1.1a	10.5 ± 1.0a	13.2 ± 3.2a
NY	5.5 ± 0.6b	3.2 ± 0.4b	1.8 ± 0.3ab	13.4	53.3 ± 8.9d	2.7 ± 0.7b	1.3 ± 0.3c	13.0 ± 0.5c	3.5 ± 1.4c
PL	5.6 ± 0.6b	3.4 ± 0.6b	1.7 ± 0.3ab	13.9	81.0 ± 8.6ab	4.1 ± 0.8ab	2.6 ± 0.3ab	12.6 ± 0.4bc	10.6 ± 2.8ab
YL	5.2 ± 0.6b	3.2 ± 0.4b	1.7 ± 0.2b	11.4	70.3 ± 10.8bc	3.5 ± 0.8ab	2.4 ± 0.7abc	12.5 ± 0.7bc	8.6 ± 3.2abc
Means	5.95	3.49	1.71	20.02	75.21	3.69	2.23	12.54	8.57
Populations	GI	GIT (d)	GD (d)	CG	VI	CN	CL (mm)	H (mm)	D (mm)
BX	3.2 ± 0.6cd	9.0 ± 1.6ab	13.2 ± 4.0a	7.5 ± 0.2c	65.1 ± 32.3c	6.8 ± 1.0b	25.6 ± 10.4b	22.2 ± 6.7bc	0.98 ± 0.19b
CY	4.2 ± 0.4bc	8.8 ± 0.8ab	11.8 ± 1.9a	7.7 ± 0.5c	76.0 ± 21.2bc	6.5 ± 0.7b	24.8 ± 8.5b	18.5 ± 4.9cd	0.87 ± 0.15b
KD	2.6 ± 0.5d	9.4 ± 1.1b	12.0 ± 3.1a	7.2 ± 0.3c	47.6 ± 26.1c	6.6 ± 1.2b	22.3 ± 8.0b	15.9 ± 5.8d	0.91 ± 0.24b
LX	4.9 ± 0.4ab	7.2 ± 0.8ab	13.6 ± 4.5a	8.8 ± 0.5ab	123.4 ± 32.8ab	8.6 ± 2.0a	37.6 ± 10.9a	25.6 ± 4.7ab	1.27 ± 0.28a
MK	5.6 ± 0.6a	6.6 ± 0.6a	14.4 ± 4.3a	9.6 ± 0.9a	146.2 ± 19.5a	9.3 ± 1.2a	44.8 ± 13.9a	27.3 ± 5.2a	1.38 ± 0.29a
NY	2.6 ± 0.5d	8.6 ± 1.7ab	11.6 ± 2.7a	7.7 ± 0.3c	44.4 ± 13.1c	6.6 ± 0.9b	26.4 ± 4.4b	16.3 ± 5.3d	0.96 ± 0.20b
PL	4.0 ± 0.6c	8.2 ± 1.3ab	12.0 ± 3.3a	7.9 ± 0.3bc	66.1 ± 27.3c	7.1 ± 1.2b	24.5 ± 6.6b	17.2 ± 6.2d	1.03 ± 0.24b
YL	3.5 ± 0.7cd	8.6 ± 1.5ab	12.0 ± 3.6a	8.0 ± 0.5bc	54.9 ± 23.1c	6.9 ± 1.0b	22.8 ± 8.0b	16.5 ± 4.3d	0.90 ± 0.13b
Means	3.84	8.30	12.58	8.06	77.96	7.30	28.61	19.94	1.04

The abbreviations are the same as in Table 2. Data are the Means ± 1 SD of 5 replications. Means followed by the same letter were not significantly different by Tukey's test (P > 0.05). For ease of viewing, some tabulated data was rounded, but Tukey's ranks are shown as calculated with unrounded data

<sup>a</sup> Population abbreviations as in Table 1

**Table 4** Differences among populations for count of seeds germinated on day 9, 14, and 26

Population <sup>a</sup>	Day 9 count <sup>b</sup>	Population	Day 14 count	Population	Day 26 count
MK-I	25.6a	LX-I	50.6a	MK-I	54.6a
LX-I	14.6ab	MK-I	49.6ab	CY-I	53.8ab
YL-III	3.2bc	PL-II	42.8abc	LX-II	53.8abc
PL-II	2.8c	CY-II	42.4abcd	PL-II	48.6abc
CY-II	1.6cd	YL-III	35.8abcd	YL-III	42.2cd
BX-I	1.2d	BX-I	32.6cde	BX-I	41.6cd
NY-III	1.2d	NY-III	25.0e	KD-II	34.4d
KD-II	0.6d	KD-II	23.4e	NY-III	32.0d

Means followed by the same letter were not significantly different by Tukey's test ( $P \leq 0.05$ )

<sup>a</sup> Population abbreviations as in Table 1, followed by provenance as determined by Gao et al. (2012)

<sup>b</sup> Count is number of seeds out of 60 germinated averaged over five blocks

At day 9, provenance was highly significant ( $P = 0.0014$ ), as was the interaction term (TSW\*provenance). Because of extreme heteroscedasticity in the covariate, provenance LSMEANS were calculated separately at four levels of TSW (see Materials and methods). This means that the effect of seed weight on germination count was quite different for each provenance. Nevertheless, on day 9 at all four tested covariate levels (overall mean seed weight and the mean seed weight of each provenance) mean germination counts ranked as Provenance I > Provenance II > Provenance III. At high seed weights, differences among provenances were most pronounced; at lower seed weights, however, such as those typical of Provenance III, there was no significant difference between germination counts of any of the provenances. Results on day 14 (early Stage III), provenance and TSW\*provenance effects were again highly significant ( $P = 0.0023$  and  $P = 0.0034$  respectively). As at day 9, at high and average TSWs, Provenance I had significantly higher germination counts than Provenance II, which in turn had higher counts than Provenance III. At lower seed weights, typical of those of Provenances II and III, there was no statistically significant difference in germination counts among provenances on tested days. The analytical model did not converge for any days later than day 14, so for day 26 we analyzed provenance effects without TSW as a covariate but with populations nested within provenances. On day 26, both provenance and population effects were highly significant ( $P = 0.0001$  and  $P = 0.0017$  respectively); provenance means ranked I > II > III, and differences between all provenances were significant ( $P < 0.05$ ).

### Variation among populations in seed germination and early seedling growth

Differences among populations for many of the seed germination traits were obvious (Table 2). The provenances and populations differed significantly for nearly all of the 13

seed germination and seedling traits we measured, the only exception was GD, which was about 3 weeks in all cases (Tables 2, 3; Fig. 2). Mean GV varied from 3.49 to 13.18 for the eight *P. densata* populations (Table 3), and the average was 8.57, which was lower than that of *P. yunnanensis* (GV = 10.10) and *P. tabuliformis* (GV = 11.92) (unpublished data) under the same experimental conditions. GV for Provenance I was 10.82, significantly ( $P = 0.0003$ ) greater than GV for Provenances II and III, which did not differ (means = 8.01 and 6.06, respectively). MK and LX populations from the northeastern periphery of the *P. densata* range showed GV values 277.7 and 255.6 % greater than the NY population. Similarly, provenances and populations differed widely and significantly for GI. Provenance I showed higher GI than Provenance II (4.60 > 3.60,  $P \leq 0.001$ ), and Provenance II was greater than Provenance III (3.60 > 3.05;  $P \leq 0.001$ ). The value of GI for the MK and LX populations was twice that of the NY population. PV was also different among provenances ( $P = 0.0087$ ) with Provenance I consistently larger than Provenance II, which in turn was greater than Provenance III. Other germination traits, including MDG and CG, were also different among provenances. Values for each trait were maximal in the MK and LX populations followed by CY and PL populations.

Seedling morphology and growth was significantly different among populations, as reflected in differences in CN, CL, seedling height (H) and ground line diameter (D), which varied from 6.48 to 9.28, 22.32 to 44.84, 15.92 to 27.28, and 0.87 to 1.38 mm, respectively (Table 3). The MK and LX populations had an average CL of  $44.84 \pm 13.85$  and  $37.64 \pm 10.85$  mm and averaged  $9.28 \pm 1.2$  and  $8.56 \pm 2.0$  cotyledons, respectively. These values were significantly greater than all other populations of *P. densata*. After 2 months of growth, the MK and LX populations had an average height of  $27.28 \pm 5.2$  and  $25.56 \pm 4.7$  mm and an average ground-line diameter of  $1.38 \pm 0.3$  and  $1.27 \pm 0.3$  mm, respectively. By contrast, NY had an average height of  $16.32 \pm 5.25$  mm and an

**Table 5** Pearson correlations between seedling germination traits, population source and climatic parameters

Traits	Longitude	Latitude	Elevation	PREC8	TMAX12	TMIN12	BIO2	BIO7
SL	0.333	0.619	-0.500	-0.738*	0.192	-0.762*	0.810*	0.762*
SW	0.506	0.735*	-0.723*	-0.639	0.309	-0.494	0.663	0.699
SLW	0.060	0.419	-0.180	-0.755*	-0.090	-0.922**	0.790*	0.743*
TSW	0.643	0.810*	-0.738*	-0.524	0.072	-0.643	0.548	0.619
GP	0.455	0.311	-0.252	-0.036	0.759*	-0.252	0.503	0.263
MDG	0.548	0.357	-0.286	-0.071	0.719*	-0.238	0.524	0.310
PV	0.429	0.476	-0.452	-0.262	0.778*	-0.286	0.619	0.476
MGT	-0.095	-0.357	0.238	0.667	-0.695	0.476	-0.881**	-0.690
GV	0.476	0.452	-0.405	-0.190	0.719*	-0.333	0.595	0.429
GI	0.500	0.381	-0.310	-0.095	0.755*	-0.286	0.548	0.333
GIT	-0.108	-0.419	0.275	0.743*	-0.518	0.647	-0.934**	-0.778*
GD	0.805*	0.878**	-0.878**	-0.195	0.454	-0.220	0.317	0.464
CG	0.095	0.357	-0.238	-0.667	0.695	-0.476	0.881**	0.690
VI	0.619	0.476	-0.429	-0.071	0.635	-0.310	0.476	0.310
CN	0.357	0.690	-0.667	-0.643	0.503	-0.452	0.714*	0.738*
CL	0.405	0.571	-0.405	-0.667	0.132	-0.690	0.690	0.619
H	0.690	0.595	-0.619	-0.214	0.527	-0.286	0.429	0.310
D	0.524	0.881**	-0.786*	-0.667	0.072	-0.690	0.643	0.810*

*PREC8* average monthly precipitation in August, *TMAX12* average monthly maximum temperature in December, *TMIN12* average monthly minimum temperature in December, *BIO2* the mean of all the weekly diurnal temperature ranges, *BIO7* Temperature annual range (Max temperature of warmest month—min temperature of coldest month). The remaining abbreviations are the same as in Table 2

Symbols \* and \*\* indicating significant differences at 0.05 and 0.01 probability levels, respectively

Red highlight indicates correlation  $\geq 0.9$ ; green indicates correlation  $0.9 > r \geq 0.8$ ; yellow indicates correlation  $0.8 > r \geq 0.7$

average ground-line diameter of  $0.96 \pm 0.20$  mm. Although PL population exhibited lower GP than CY, the ground-line diameter of PL seedlings after 2 months was greater than CY seedlings, as was also true of seeds and seedlings of the Baoxing (BX) population.

Seedling vigor, as measured by the seedling growth VI, was also different among provenances (Table 2), with Provenance I (mean = 111.58) greater than Provenance II (63.25), which was higher than Provenance III (49.61). The MK and LX populations had the highest VI, which showed earlier GIT and shorter MGT than other populations.

### Correlations between traits

All measured traits except GIT and MGT were positively correlated with longitude and latitude (Table 5). SW and TSW showed a strong positive correlation with latitude ( $r = 0.735$ ,  $P = 0.038$  and  $r = 0.810$ ,  $P = 0.015$ , respectively). GD was positively correlated with longitude ( $r = 0.805$ ,  $P = 0.016$ ) and latitude ( $r = 0.878$ ,  $P = 0.004$ ). There was a strong and significant positive correlation between seedling ground line diameter and latitude ( $r = 0.881$ ,  $P = 0.004$ ). Most measured traits were negatively correlated with elevation. For example, SW, TSW, GD, and seedling ground line diameter were

significantly and strongly negatively correlated with elevation ( $r = -0.723$ ,  $-0.738$ ,  $-0.878$  and  $-0.786$ , respectively). Seeds that germinated quickly (low GIT) were associated with sites with high weekly diurnal temperature ranges (*BIO2*) ( $r = -0.934$ ) and seeds with low SLW were strongly associated with locations with warmer minimum temperatures in December ( $r = -0.922$ ). We also determined correlations between seed germination and seedling growth and specific climatic parameters, including maximum temperature, minimum temperature, precipitation in each month, and 19 other bioclimatic variables, for eight populations of *P. densata*. Mean diurnal range (*BIO2*) was highly correlated with SL ( $r = 0.810$ ), GP ( $r = 0.503$ ), seedling height ( $r = 0.429$ ) and ground line diameter ( $r = 0.643$ ), and highly negatively correlated with GIT ( $r = -0.934$ ) and GD ( $r = -0.881$ ). Maximum temperature in December was strongly and significantly correlated with many seed germination traits ( $r = 0.7-0.8$ , Table 5). Additionally, seed shape (seed length to width ratio) was highly correlated with minimum temperature in each month (for most months,  $P \leq 0.01$ ; data not shown). Fast germination was clearly associated with larger, heavier seeds that contained more and longer cotyledons, for example, seedling height and ground-line diameter showed high and significant correlations with seed germination and seed size ( $r = 0.8-0.9$ , Table 6).

**Table 6** Pearson correlations between seedling germination and early growth traits and seed size

Parameters	SL	SW	SLW	TSW
GP	0.630	0.641	0.381	0.633
MDG	0.655	0.674	0.392	0.648
PV	0.724*	0.740*	0.427	0.715*
MGT	-0.935**	-0.913**	-0.734*	-0.898**
GV	0.736*	0.755*	0.439	0.724*
GI	0.844**	0.833**	0.604	0.829**
GIT	-0.960**	-0.941**	-0.766*	-0.920**
GD	0.869**	0.855**	0.612	0.904**
CG	0.947**	0.925**	0.7645	0.908*
VI	0.957**	0.941**	0.699*	0.960**
CN	0.983**	0.973**	0.713*	0.970**
CL	0.989**	0.951**	0.823**	0.976**
H	0.850**	0.823**	0.635	0.888**
D	0.968**	0.941**	0.804*	0.958**

The abbreviations are the same as in Table 2

Symbols \* and \*\* indicate significant differences at 0.05 and 0.01 probability levels, respectively

Red highlight indicates correlation  $\geq 0.9$ ; green indicates correlation  $0.9 > r \geq 0.8$ ; yellow indicates correlation  $0.8 > r \geq 0.7$

## Discussion

Provenances and populations of *P. densata* differed significantly for nearly every measured trait related to seed germination and early seedling growth. Differences in germination characteristics based on provenance are commonly observed for widely distributed plant species like *P. wallichiana* (Rawat and Bakshi 2011), *Combretum aculeatum* (Bognounou et al. 2010), and *Adansonia digitata* (Asogbadjo et al. 2011). The extent to which the results reported here for *P. densata* are related to its likely hybrid origins as a species (Wang and Szmidi 1990, 1994) is not at all clear; non-hybrid species show similar patterns of provenance-level variability for seed germination and seedling growth. It is possible, nevertheless, that the provenance-level differences we observed in seed and seedling traits are explained by regional differences in the genomic mixing of the parental species (Baskin and Baskin 1973). The diversity of genetic composition among populations of *P. densata* has been investigated through the analysis of multiple marker systems. The genomic contributions from each parental species differed among sampled populations (Wang and Szmidi 1994; Wang et al. 2001; Song et al. 2002, 2003). Presumably these genomic differences reflect distinct selective pressures in the diverse habitats in the range of *P. densata* (Gao et al. 2012), and these differences may also lead to additional functional adaptation and differentiation. Gao et al. (2012) showed that the three provenances we tested belonged to

distinct genetic clusters, with the region corresponding to Provenance I serving as the original zone of hybridization between *P. yunnanensis* and *P. tabuliformis*; populations in Provenances II and III diverged from Provenance I about 6.6 million years ago. Gao et al. (2012) suggested that as *P. densata* expanded westward its demography was affected by strong bottlenecks and isolation, leading to genetic and, presumably, phenotypic divergence of the type we observed.

It was difficult to evaluate the biological basis for the relationship between longitude, latitude and elevation and the differences we observed among populations in seed and seedling traits because sample sizes were relatively small (eight populations) and because populations from higher latitudes are not necessarily adapted to lower temperatures and shorter growing seasons. As demonstrated by the strong and significant correlations between climatic variables and seed morphology, seed germination, and seedling growth traits, however, the elevation of provenances might be reflected in biologically relevant adaptation. We found that populations from environments with greater fluctuations in temperature produced larger seeds that germinated faster with higher GP, and they grew larger seedlings than seeds from provenances where temperatures were more stable. Similarly, temperature was correlated with seed morphology; we observed a very high and significant correlation between the minimum temperature in each month at the seed source and seed shape (seed length to width ratio). Thus, seeds from colder provenances were shorter and rounder (had less surface area) than those from warmer provenances. These differences could be attributed to any number of important adaptive factors, including seed predators and relative humidity. Surprisingly, many traits related to the speed and completeness of germination were strongly correlated with maximum temperature in December. If this association can be further validated, it may improve our ability to predict the germination capacity of seed lots based on the environment where they were harvested.

The strong relationship we observed between seed size and germination was not surprising, but rapid and complete germination of seeds is only one component of reforestation success when aerial seeding is employed. The phenology of seedling growth and bud-set, frost and drought resistance, as well as local conditions such as the abundance of herbivores and the number and types of weeds, can all strongly influence establishment. It is not safe to assume that local seeds will always be the best, as adaptation can lag behind environmental change. Detailed studies using reciprocal transplantation might be necessary to determine which seed sources are best for any regeneration location.

Seed size and weight had pronounced effects on seed germination, but the effect of seed size on germination traits varied by provenance. It would be useful in future experiments to sort seeds by size and to retain their source identity in order to measure within and among population variance for seed size, and to determine if seed size and shape have different adaptive functions in different environments. In this study, a significant and positive correlation was found between rapid germination and seedling growth. It was observed that *P. densata* seed sources that had higher values for SL, width, and weight, also showed faster germination and higher percent germination. Similar results were reported for loblolly pine by Dunlap and Barnett (1983). (Houle and Filion 1993) pointed out that larger (i.e., heavier) seeds of *P. banksiana* tended to germinate faster. The vast majority of seed germination studies assume that seed size and weight have pronounced effects on seed germination (Rawat et al. 2006). Seed size and seed mass are not exactly the same, but they are closely related in many conifer species as they were in our study ( $r = 0.905$ ). Large seeds draw upon a greater nutrient pool than small seeds, and as a result typically germinate at a higher rate than small seeds (Kandya 1978). Large *P. densata* seeds may also have larger embryos containing more cells than their smaller counterparts, resulting in bigger seedlings. Venable and Brown (1988) suggested that under favorable conditions seedling establishment is more or less independent of seed size, but under unfavorable conditions only large-seeded plants can successfully establish. Nevertheless, in our study, seeds from higher and colder provenances were the smallest, possibly due to nutrient limitation, but as previously mentioned, environmental factors such as predation, dormancy and dispersal can produce disruptive selection for seed size (Venable and Brown 1988).

We observed a weak correlation between GP and seedling growth. For example, seeds of the BX population had low percent germination but high seedling height, and seeds of the CY population had high percent germination but low seedling height. A similar lack of correspondence between percent germination and subsequent growth was reported by Johnston et al. (2003) for *Iris* species. It is possible the low correlation we observed between percent germination and seedling growth was associated with elevation of population origin. *P. densata* exhibited decreasing GP and speed with an increase in the elevation of seed sources, which is a common phenomenon in other pine trees (Harry et al. 1983; Isik 1986).

The growth variation we observed could be also associated with latitude of population origin ( $r = 0.881$ ). MK and LX populations from the highest sampled latitude had higher seed germination and seedling growth than all other samples. Conversely, the lowest latitude population (NY) showed low seed germination and seedling growth.

Hannerz and Westin (2000) found that *Picea abies* growth traits were highly correlated to the latitude of the maternal stand; latitude explained 49 to 87 % of the variation among stands in the analyzed growth traits at two nurseries.

The observed variation among *P. densata* populations can be used to guide afforestation and regeneration of *P. densata*, as well as nursery practices. Provenances with high germination rates and strong early growth could be grown together in the nursery or sown together by aerial seeding. For example, MK and LX populations can be planted together in seedbeds for efficient management because these populations had similar seed germination and early seedling growth. Likewise, populations that had faster germination and better seedling performance can be planted in areas with high competition from shrubs or predation by rodents or birds to accelerate regeneration and reduce risk. Seeding rate is important for nurseries and for aerial seeding; populations with high seed germination and seedling growth (i.e. MK and LX) can be planted at lower densities than those with lower rates of germination—of course proper match between seed sources and regeneration sites is always critical.

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