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Hsin-Wu Hsu

Temperature Dependence of Tree Reproductive Processes:  
Flowering Phenology, Pollen Viability, and Seed Germination

Hsin-Wu Hsu

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Reading Committee:

Soo-Hyung Kim, Chair

Jonathan Bakker

Gregory Ettl

Program Authorized to Offer Degree:  
School of Environmental and Forest Sciences

University of Washington

**Abstract**

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Hsin-Wu Hsu

Chair of the Supervisory Committee:

Soo-Hyung Kim

School of Environmental and Forest Sciences

Many plant species are already shifting their geographic ranges in response to climate change. Species range shifts in response to temperature are conditioned by many factors. Reproductive phases, e.g., flowering phenology, pollen germination, pollen tube growth, fruit set, and seed germination, have proven to be the most temperature-vulnerable stages. Therefore, the impact of climate change on reproduction could be a major limitation on future tree distribution. Some researchers indicate that all species distribution models have to integrate or improve species dispersal and migration processes to be able to provide accurate projections of future distributions. However, species distribution models rarely consider temperature-induced reproductive disorder and failure, e.g., mismatches with pollinators or seasonality and inability

of pollen to germinate, because this species-specific information is hard to obtain for trees. My study used Yoshino cherry (*Prunus x yedoensis*) blossom data in Japan to demonstrate that phenological changes in flowering caused by higher-than-normal temperatures may induce reproductive disorders and further impact their distribution. We will be able to apply the same method to investigate the warming effects on male and female cones with increasing flowering phenological observations of conifer species in the future. In addition, I utilized the pollen viability test on conifer species, *Pinus contorta* Dougl. ex. Loud., *Picea engelmannii* Parry ex Engelm., and *Pinus ponderosa* Dougl. ex Laws., as a method to study temperature effects on reproductive success and seed germination tests on the same three conifer species plus *Pseudotsuga menziesii* (Mirb.) Franco to understand the possibility of seedling regeneration rate under climate change. My goals are to (1) evaluate the effects of winter warming versus spring warming on flowering phenology and how warming in these different periods exerts variable effects on flowering dates; (2) explore how temperature dependence of pollen germination and tube growth vary among conifer species and populations of the same species collected from various elevations; (3) examine how temperature dependence of seed germination varies among different conifer species and populations of the same species collected from various elevations and whether the variation of germination traits relates to any climate factors at seed-collecting sites. I found that rates of change in Yoshino cherry bloom dates differ significantly between before and after 1980, and excessive warming could delay blooms, which has manifested at low latitudes. Optimal temperature and temperature ranges for pollen germination and tube growth vary among species and elevations within the same species and reflect the acclimation or adaptation of species to spring mean temperatures.

Seeds from different species and different elevations within the same species had different germination traits correlated with their ecological niche, and germination traits do not show the environmental gradients. The results provide the exact optimal temperatures for pollen germination, tube growth, and seed germination that people always suspect but never know the values. These findings also provide the information needed for improving current conifer species distribution models based on reproductive biology and contribute to more effective climate adaptation strategies, e.g., seed transfer, assisted migration, locating corridors and refugia, etc., for policymakers and stakeholders to help mitigate climate change impacts, and increase our understanding of forest resilience.

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# Chapter 1

## Introduction

The global climate is changing rapidly due to human activities, leading to unprecedented impacts on ecosystems, ecological processes, and biological events (Bykova et al., 2012; IPCC, 2014b). Temperature is a key climate variable that dictates ecological processes and biological events and is expected to increase globally from 1.4°C (low GHG emission scenario) to 4.4°C (very high GHG emission scenario) with frequent temperature extremes by the end of this century (Calvin et al., 2023). Temperatures can affect plant reproduction during a variety of developmental stages. It is nearly impossible to investigate the temperature effects on all of these developmental stages of trees because it is hard to apply temperature treatments on large trees. This makes running a controlled temperature experiment on tree reproduction quite difficult, making species-specific reproduction information hard to obtain. Reproductive phases, e.g., flowering phenology, pollen germination, pollen tube growth, fruit set, and seed germination, have proven to be the most temperature-vulnerable stages (Bykova et al., 2012; Hedhly et al., 2009; Kakani et al., 2005; Zinn et al., 2010). Therefore, the impact of climate change on reproduction could be a major limitation on tree distributions. However, species distribution models rarely consider temperature-induced reproductive disorder and failure (Bykova et al., 2012), e.g., mismatches with pollinators or seasonality and inability of pollen to germinate, etc. I proposed investigating the mismatch of flowering phenology, pollen viability, and seed germination in response to temperature to discuss the potential effects of reproductive disorder and failure on the species distribution model under climate change.

There is ample evidence that the spring phenology of plants has advanced during the past decades (Abu-Asab et al., 2001; Cleland et al., 2007; Cook et al., 2012; Ettinger et al., 2020; Fu et al., 2014; Menzel et al., 2006; Parmesan, 2007; Primack et al., 2009). Some notable exceptions exist to these observations (Kozlov & Berlina, 2002; Yu et al., 2010, 2012) and have exhibited a delay in spring leaf-unfolding despite the general rapid warming trend observed in recent years. Some studies have suggested that these contradicting trends (i.e., acceleration or

delay in response to warming) are attributable not only to species differences but also to the timing of warming (Asse et al., 2018; Wenden et al., 2020), hinting at potentially complex interactions among factors (Ettinger et al., 2020; Flynn & Wolkovich, 2018; Kaufmann & Blanke, 2019; Kim et al., 2022; Wenden et al., 2020). A plausible hypothesis is that winter warming may lead to insufficient chilling required for breaking endodormancy (Asse et al., 2018; Chuine et al., 2010; Yu et al., 2010; Yun et al., 2017), which could delay or even compromise the onset of spring events (Chuine et al., 2016), e.g., flowering and leaf-unfolding. This could explain the exceptions to the common trend in which spring warming advances budburst and flowering through a rapid accumulation of growing degree days during the forcing period once endodormancy is released. In Chapter 2, I test this hypothesis with historical bloom data of Yoshino cherry (*Prunus x yedoensis*) collected from multiple locations in Japan across a latitudinal gradient. The historical records of cherry blossoms in Japan provide a unique opportunity to test the effects of warming in different seasons on the phenology of temperate trees. Long-term phenological data on tree reproductive biology are rare, especially on conifer male and female cones. Therefore, we used Yoshino cherry data to demonstrate the effects of temperature on the flowering phenology. With increasing flowering phenological observations of conifer species in the future, we will be able to apply the same method to investigate the warming effects on male and female cones to see whether warming will create a mismatch in conifers and subsequently, affect seed production and species distribution.

When high-temperature stress is applied separately on male and female gametes before pollination, it is frequently observed that pollen is often the most vulnerable (i.e., immature and ungerminated pollen, shorter pollen tube length or unsuccessful fertilization) and weakest link in the reproductive cycle (Zinn et al., 2010). The ability and speed of pollen germination and the rate of pollen tube growth under different temperatures are traits that can be selected for increasing the probability of successful fertilization by pollen from a particular donor (Pasonen et al., 1999, 2000, 2001, 2002; Skogsmyr & Lankinen, 2002) and consequently, shape the genetics structure and adaptation of the next generation. In addition, the flowering phenology changed due to warming, which means pollen will be exposed to different temperature regimes. Therefore, determining the temperature dependence of conifer pollen germination

and pollen tube growth from different conifer species and populations is essential to evaluate species' response to temperature for predicting and mitigating the impacts of climate change on plant populations and ecosystems, but is rarely studied in conifers. Conifer pollen and pollen tubes exhibit numerous distinctive traits absent in flowering plants, and these distinctions represent a significant evolutionary diversion in the development of male gametophytes (Fernando et al., 2005). We do not know whether these trait differences will bring up various temperature responses in conifers. Thus, the study of pollen germination and tube development in conifers not only provides valuable insights into a more rudimentary form of sexual reproduction but also enhances our understanding of this crucial phase in the reproductive cycle of conifers and seed plants. In Chapter 3, I aim to explore the research questions of (1) how temperature dependence of pollen germination and tube growth varies among different conifer species and populations of the same species collected from various elevations, (2) whether the optimal temperatures for pollen germination and tube growth are correlated to pollen collection elevations or to the temperatures when pollen sheds, and (3) whether the optimal temperatures of pollen germination and tube growth are the same, different or correlated. The results can advance our understanding of how conifer pollen responds to warming and provide valuable information for enhancing the projection of conifer species' distribution models under climate change.

Seed germination is an essential process in the plant life cycle. It affects seedling survival and establishment and determines population persistence, community structure, and species distribution (Bykova et al., 2012; Cochrane et al., 2015). Seed germination traits have been overlooked compared to frequently used morphological traits, e.g., seed mass, shape, size, etc. They should be incorporated into vegetation science to include community ecological functions not demonstrated solely by adult plant traits (Jiménez-Alfaro et al., 2016). Temperature is one of the most critical environmental factors that regulate seed germination speed, uniformity, and percentage. It affects the rate of chemical reactions and the enzyme activity that controls various seed metabolic processes. In addition, optimal germination temperatures are determined by the conditions the seeds have adapted to in their natural environment (Chamorro et al., 2018). Therefore, the specific temperature requirements for germination vary

depending on the plant species (Baskin & Baskin, 2014). Warmer temperatures may promote faster seed germination for species at high elevations, inhibit germination, or make seeds difficult to survive for species at low elevations. Some studies have reported that germination response to environmental conditions within species could be quite different due to intraspecific variation (Chamorro et al., 2017; Cochrane et al., 2015). This variation is also key to mitigating the species' vulnerability to changing climate and providing species adaptation and conservation opportunities (Chamorro et al., 2017; Cochrane et al., 2015; Marcora et al., 2008; Messier et al., 2010; Sides et al., 2014; Wu et al., 2018). In Chapter 4, I explore the research questions of how temperature dependence of seed germination varies among different conifer species and populations of the same species collected from various elevations and whether the variation of germination traits relates to any climate factors at seed-collecting sites. The results could provide valuable information for seed transfer, assisted migration, and forest management and predict population dynamics, community structure, and range shifts under climate warming.

An important limitation in most species range shift studies is that realistic species displacement rates are not accounted for (i.e., rates at which species can shift their ranges through reproductive success, dispersal, establishment, and maturation). Ecological Niche Models (ENMs) are used in most projections of climate change impacts on future species range shifts based on correlative models of current relationships between environmental factors and species distribution. They only indicate changes in the location of favorable and unfavorable climates, from which potential shifts in species distribution can be inferred, but not the rate of change. That is, they do not consider the myriad of biological processes that would take place under a geographic shift. In addition, they assume that there is no intra-species variability and that species produce homogeneous responses to climate variation across species' ranges (Benito Garzón et al., 2011). Their prediction accuracy is usually based on the training data and potentially extrapolated future distribution from the current trend that could raise concerns. Process-based models are increasingly used to predict future species distributions (IPCC, 2014a) that can incorporate rates of change, variability, and other factors besides the changing climate. Climate-associated plant phenology shifts can majorly drive species distribution (Parmesan &

Yohe, 2003; Root et al., 2003). New approaches to modeling tree responses also suggest that climate change impacts reproduction and could be a major limitation on tree distributions (Morin et al., 2007). Chuine (2010) suggested that all species distribution models have to integrate or improve species dispersal and migration processes to be able to provide accurate projections of future distributions (e.g., Midgley et al., 2007; Morin & Lechowicz, 2008; Thuiller et al., 2008). Trees can only expand their territories if climate change increases the availability of suitable sites for recruitment and a sufficient number of viable seeds arrive at the suitable sites. Therefore, the availability of viable seeds and suitable sites for germination and growth will likely constrain the shift of plant species under climate change. Most conifer trees are long-lived plants that need decades to reach reproductive maturity, reproduce, and increase abundance before further expanding their ranges (Platt et al., 1988). If the climate change velocity is too fast, the suitable sites that climate change created are no longer available when the tree species eventually reach the site. Thus, understanding temperature effects on tree reproduction and recruitment is essential to predicting range shifts but rarely taken into account (Bykova et al., 2012). Moreover, many other factors, e.g., growth, mortality, phenotypic plasticity, genetic variability, resilience, etc., also influence the species' future persistence, not only reproduction matters. Hence, in Chapter 5, I suggest several future directions and propose an improved process-driven species distribution model incorporating not only temperature effects on reproductive success and recruitment but also seedling growth, mortality, and genetic variability to enhance projective accuracy and answer more questions. The results can contribute to more effective climate adaptation strategies, e.g., seed transfer, assisted migration, locating corridors and refugia, etc., for policymakers and stakeholders, help mitigate climate change impact, and increase our understanding of forest resilience.

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## Chapter 2

### Variable warming effects on flowering phenology of cherry trees across a latitudinal gradient in Japan

**Authors:**

Hsin-Wu Hsu<sup>1</sup>,

ORCID: 0000-0002-6347-4540

E-mail address: hwhsu@uw.edu

Kyungdahm Yun<sup>1, 2</sup>,

ORCID: 0000-0003-2289-4386

E-mail address: kdyun@uw.edu; kdyun@jbnu.ac.kr

Soo-Hyung Kim<sup>1</sup>,

ORCID: 0000-0003-3879-4080

E-mail address: soohkim@uw.edu, Corresponding author

<sup>1</sup>School of Environmental and Forest Sciences, University of Washington, Seattle, WA 98195-4115, USA

<sup>2</sup> Department of Smart Farm, Jeonbuk National University, Jeonju 54896, Republic of Korea

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## Abstract

Rising global temperatures are affecting plant phenology. Spring warming can lead to early flowering due to accelerated heat accumulation after dormancy. Winter warming, however, may counteract this acceleration by extending the chilling period required for endodormancy release. We tested this hypothesis with historical bloom data of a flowering cherry (*Prunus x yedoensis*) collected from multiple locations in Japan across a latitudinal gradient. We determined differential chilling and forcing temperature effects based on advances (-) or delays (+) of bloom dates per degree Celsius of change (temperature sensitivity,  $S_T$ ). We applied a chilling-forcing modeling approach to reveal and disentangle the differential temperature effects during and after dormancy on spring phenology across different latitudes over time. Our analysis showed that the effects of chilling temperatures during dormancy were variable along the latitudinal gradient, while the effect of forcing temperatures after dormancy was more consistent regardless of latitude. Notably, the elevated temperatures, and thereby reduced chilling, during endodormancy delayed the actual bloom dates at lower latitudes, and this trend was more pronounced in recent years. Our results support the hypothesis that the effect of winter warming during endodormancy can offset or even outweigh the effect of spring warming by extending the dormancy period, and this can manifest as an overall delay in bloom dates. While the delay in flowering phenology due to winter warming was observable currently only in lower latitudes, it is likely to expand to higher latitudes in the future climate if the warming trend continues. Our findings also highlight the value of concerted long-term phenological records that can provide insights into the mechanisms underlying plasticity in tree physiology with a unique opportunity to predict their responses in a changing climate.

## 2.1 Introduction

The global climate is changing rapidly as a consequence of human activities leading to unprecedented impacts on ecosystems, ecological processes, and biological events (Bykova et al., 2012; IPCC, 2014). Temperature is a key climate variable that dictates ecological processes and biological events. Tree-ring and pollen-based temperature reconstruction studies (Ahmed et al., 2013; Viau et al., 2012; Wahl & Smerdon, 2012) suggest that recent decades have been the warmest across temperate North America and Asia over the past 500 years or more. This warming trend is consistent with the observed advances in plant phenology (Campoy et al., 2011). During the past decades, considerable shifts in tree phenology have been reported worldwide. There is ample evidence that the spring phenology of plants has advanced during this warming period (Abu-Asab et al., 2001; Cleland et al., 2007; Cook et al., 2012a; Ettinger et al., 2020; Fu et al., 2014; Menzel et al., 2006; Parmesan, 2007; Primack et al., 2009). Some notable exceptions exist to these observations. For example, vegetation at high altitudes (Yu et al., 2010, 2012) and in northern latitudes (Kozlov & Berlina, 2002) have exhibited a delay in spring events despite the general rapid warming trend observed in recent years. Some studies have suggested that these contradicting trends (i.e., acceleration or delay in response to warming) are attributable not only to species differences but also to the timing of warming (Asse et al., 2018; Wenden et al., 2020), hinting at potentially complex interactions among multiple factors (Ettinger et al., 2020; Flynn & Wolkovich, 2018; Kaufmann & Blanke, 2019; Kim et al., 2022; Wenden et al., 2020). A plausible hypothesis is that winter warming may lead to insufficient chilling required for breaking endodormancy (Asse et al., 2018; Chuine et al., 2010; Yu et al., 2010; Yun et al., 2017), which could delay or even compromise the onset of spring events (Chuine et al., 2016). This could explain the exceptions to the common trend in which

spring warming advances budburst and flowering through rapid accumulation of growing degree days during the forcing period once endodormancy is released.

Long-term phenological data on reproductive biology in trees are rare. The historical records of cherry blossoms in Japan and elsewhere provide a unique opportunity to test the effects of warming in different seasons on the phenology of temperate trees. Many countries around the world have a rich history of planting ornamental cherries and celebrating their blossoms as a symbol of spring. The literature in Japan, in particular, indicates that the first cherry blossom festival was celebrated in Kyoto as early as the 9th century (Primack & Higuchi, 2007; Taguchi, 1939). The cherry blossom festivals symbolize important historical and cultural values of many celebrated cherry tree varieties (Jefferson & Fusonie, 1977; Primack & Higuchi, 2007; Sakurai et al., 2011); Japanese mountain cherry (*Prunus jamasakura*) and Somei-Yoshino (*Prunus x yedoensis*) are among the most celebrated and widely planted cherry tree varieties in Japan (Sakurai et al., 2011). The cherry blossom festivals have enriched art, music, and literature (Nagai et al., 2019) and provided economic and social benefits to the local communities (Sakurai et al., 2011). Moreover, the historical records of these festivals are a unique and critical source of information for reconstructing the past climate and its variability going back to the 9th century (Aono & Kazui, 2008). While the record of cherry blossoms and their festivals goes back to centuries ago, it wasn't until around 100 years ago that more organized phenological observations were collected, along with matching temperature data from nearby weather stations (Aono & Kazui, 2008).

The phenological data suggest that cherry trees have been flowering earlier in the past several decades than in the previous 1200 years in Kyoto, Japan, and other locations worldwide (Abu-Asab et al., 2001; Aono & Kazui, 2008; Miller-Rushing et al., 2007). The study of cherry

blossom phenology is important not only because of its cultural and economic significance but also because it relates to long-term ecological changes and ecosystem services as a harbinger of the current climate change and its impacts (Allen et al., 2014; Chung et al., 2011; Nagai et al., 2019; Sakurai et al., 2011). For example, a potential vulnerability of early flowering caused by climate change is that it could create a phenological mismatch between plants and insects, including pollinators, whose phenological synchrony is critical for their survival and the ecological services they provide. This mismatch could further affect tree reproduction, including the production and quality of seeds and fruits and, thus, their regeneration potential.

Endodormancy and ecodormancy play important roles for most temperate trees to regulate their dormancy and prevent buds from growing when they encounter a brief period of favorable cool but not freezing cold temperatures during winter (Asse et al., 2018). A few weeks to months of cold temperature are required to break this physiological state, termed endodormancy (Lang et al., 1987). Subsequently, ecodormancy starts as the chilling requirements to break endodormancy are fulfilled, e.g., 22.3 to 79.3 Chill Portions (CP), depending on the varieties of sweet cherries in Germany (Kaufmann & Blanke, 2017). During the ecodormancy period, forcing above a certain temperature threshold is critical for accumulating heat units. As buds experience temperatures above the threshold, the ecodormancy is released to reach budburst, e.g., 3,473 growing degree hours (GDH) for 'Schneiders' sweet cherry in Germany (Luedeling et al., 2013a). However, the effect of warmer than usual temperatures on dormancy phenology is likely to be variable depending on whether a tree is at the endodormancy, ecodormancy, or post-ecodormancy (Luedeling, 2012). The increasing temperature during the forcing phase generally advances budbreak and flowering, while warming during the chilling phase may advance, delay, or have no effect on budburst and

flowering (Guo et al., 2013; Luedeling & Gassner, 2012). If chill units accumulate more slowly during the endodormancy period, their chilling requirements may be fulfilled later, delaying the phase to accrue heat units and ultimately leading to a later bloom or leaf emergence date. The delaying effect of winter warming may be moderated if warming also occurs in the forcing phases; that is, the “counter” effect of warming during the chilling period is most likely to be prominent when a warm winter is not followed by a warm spring (Harrington et al., 2010; Harrington & Gould, 2015; Luedeling, et al., 2013b). Thus, investigating the anomalies between winter and spring temperatures could disentangle the variable effects of warming between chilling and forcing phases.

The delaying effect of warming during the chilling period on spring phenology has been hinted at in some studies (Asse et al., 2018; Fu et al., 2015). For example, Fu et al. (2015) indicated that the rate of recent advancements in spring phenology appears to be slowing down in European regions. However, observational or experimental studies that have evaluated these counteracting forces systematically remain rare to this date. The long-term records of cherry blossom dates across a latitudinal gradient in Japan present a unique dataset to examine the effects of winter warming on spring phenology and flowering time associated with endodormancy release. Flowering cherry trees are considered an early indicator of climate impacts because their flowering time is highly sensitive to temperature changes (Miller-Rushing et al., 2007). Photoperiod also controls the induction and release from dormancy and the onset of growth and reproductive events such as synchronous flowering (Körner & Basler, 2010). While the interactive effects between photoperiod and temperature may lead to a dual dormancy control system in temperate species to prevent tissue damage from unexpected cold weather, most *Prunus spp.* are considered weakly sensitive to photoperiod (Heide, 2008).

Therefore, flowering cherries provide a unique opportunity to investigate the temperature effects with little photoperiodic interferences across the latitudinal gradient.

This study evaluates the effects of winter warming versus spring warming on flowering phenology along a latitudinal gradient based on historical records of cherry blooms collected in Japan over the past seven decades. We provide evidence to demonstrate that: 1) winter and spring warmings exert varying effects on cherry bloom dates across different latitudes; 2) the changes in bloom dates are driven by the net effect of chilling and forcing; 3) earlier spring events caused by warming during forcing period could be offset or overridden by strong winter warming during chilling period; and 4) a delay in bloom dates following warm winters has manifested at lower latitudes in recent years.

## 2.2 Materials and Methods

### 2.2.1 Phenology and temperature data

We used publicly available historical records of full bloom dates of the ornamental cherry cultivar, *Prunus x yedoensis* 'Yoshino', from 1953 to 2021, published by the Japan Meteorological Agency website (Last accessed on March 9, 2022). In this dataset, the full bloom date marks the time when 80% of the blossoms in sample trees are fully open. Daily minimum, average, and maximum air temperature data from weather stations at locations corresponding to the phenology observations were obtained from the Japan Meteorological Agency's Automated Meteorological Data Acquisition System (AMeDAS) (Last accessed on March 9, 2022). We selected 12 locations with full observation records from 1953 to 2021. The specific locations we chose for this work cover a latitudinal gradient from 31°N to 43°N (Figure 1.1). The

elevation of all study sites is below 20m a.s.l., except for Sapporo (40m a.s.l.). We defined that temperature data from each bloom season starts in July in the previous calendar year and ends in June. For example, the bloom season of 2021 begins in July 2020 and ends in June 2021. The split month is unlikely to impact the chilling and forcing model behaviors as long as the divided month is after bloom dates and before the dormancy when the air temperatures are mostly higher than the chilling threshold. The bloom dates in northern Japan end in late May; therefore, we defined the bloom season as starting in July (approximately a month later than the bloom dates) to ensure we did not accidentally cut the bloom season.

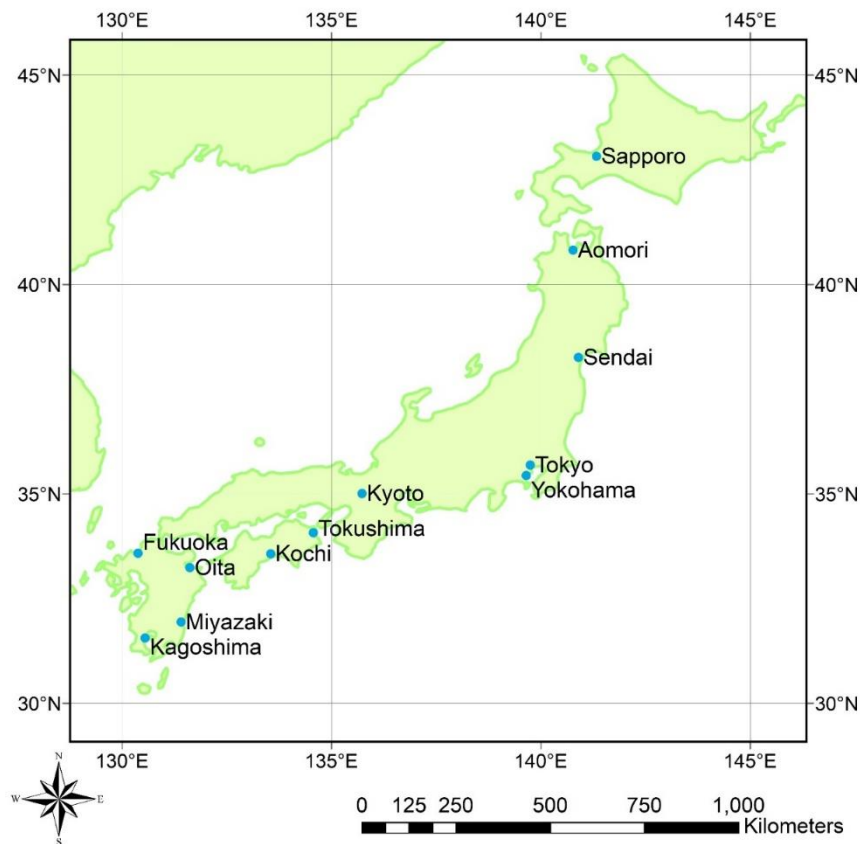


Figure 2.1 Study sites. Blue dots are cities across a latitudinal gradient from Sapporo (43.06°N) to Kagoshima (31.56°N) in Japan, where the cherry blossom records and corresponding weather data were collected from 1953 to 2021.

## 2.2.2 Identification of climatically contrasting seasonal combinations

To test the hypothesis that cherry bloom dates are affected differentially by the temperatures in chilling and forcing periods along the latitudinal gradient, we first defined chilling and forcing periods by using Partial Least Squares (PLS) regression (Benmoussa et al., 2017; Fernandez et al., 2021; Guo et al., 2015a; Guo et al., 2013; Guo et al., 2015b; Luedeling, 2012; Luedeling, et al., 2013b, 2013a; Luedeling & Gassner, 2012; Martínez-Lüscher et al., 2017; Wenden et al., 2020) to analyze the influence of daily temperatures on cherry bloom dates. Partial Least Squares (PLS) regression correlated the cherry bloom dates with daily chill and heat accumulation. It is a reliable method in situations where independent variables are highly auto-correlated and where the number of independent variables exceeds the number of observations (Luedeling et al., 2013b). We used an 11-day running mean of daily temperatures as initially proposed by (Luedeling & Gassner, 2012) and identified the start and end of each period using the positive and negative coefficients of the PLS regressions, with sufficient importance characterized by the values of the variable importance in the projection (VIP). The specific criteria we used to define chilling and forcing periods for each location except for Sendai were periods of at least five consecutive days with  $VIP \geq 0.8$  and continuously negative (forcing) or positive (chilling) coefficients (Wenden et al., 2020). For Sendai, a visual inspection of the PLS-generated patterns was needed to identify the chilling and forcing periods because the threshold of  $VIP > 0.8$  was unable to produce a biologically meaningful chilling period. In this case, we used at least five consecutive days with  $VIP \geq 0.72$  to define the end days of the chilling period. In addition, we restricted the forcing period to begin after December 1 based on the hypothesis that forcing periods cannot begin before the chilling period, as (Wenden et al.,

2020) suggested. The start and end dates of chilling and forcing periods of each location are shown in Table SA2.1.

We then calculated the average temperatures of each period (i.e., chilling and forcing) for the study period (1953-2021) and further classified these two periods into cold chilling (CC), warm chilling (WC), cold forcing (CF), and warm forcing (WF) years based on the top and bottom 50 percentile of each period. Next, we grouped individual years based on the top and bottom 50 percentiles for both the chilling and forcing periods. This process allowed us to classify all years into four different combinations: cold chilling-cold forcing (CC-CF), cold chilling-warm forcing (CC-WF), warm chilling-cold forcing (WC-CF), and warm chilling-warm forcing (WC-WF), based on their average temperatures in chilling and forcing periods (Figure 2.2). Years classified as different combinations in each location are shown in Table SA2.2. The average chilling and forcing temperatures of these combinations and all study years at different locations are shown in Table SA2.3. Likewise, the full bloom dates of Yoshino cherries were also classified into the same four groups corresponding to the chilling-forcing temperature combinations for each year, as conceptualized in Figure 2.2.

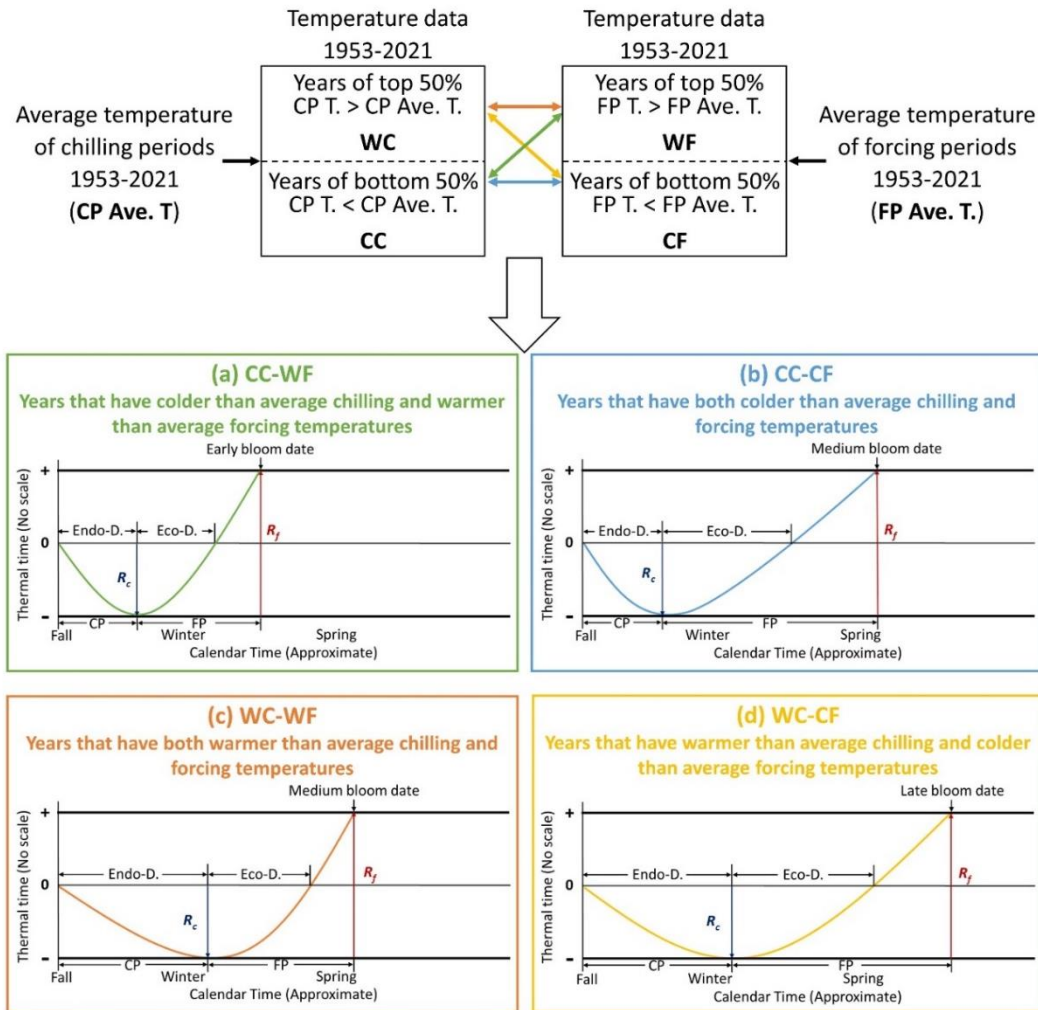


Figure 2.2 A schematic of classifying climatically contrasting seasonal combinations and conceptual models for cherry full bloom date accounting for the relationships among chilling and forcing requirements and dormancy release (Modified from Chung et al., 2011). Arrow color corresponds with category. (a) Model 1: cold chilling and warm forcing period (CC-WF); (b) Model 2: cold chilling and cold forcing period (CC-CF); (c) Model 3: warm chilling and warm forcing period (WC-WF), and (d) Model 4: warm chilling and cold forcing (WC-CF). CP: chilling period; FP: forcing period;  $R_c$ : chilling requirement;  $R_f$ : forcing requirement; Endo-D.: endodormancy; Eco-D.: ecodormancy. The y-axis shows the direction of chilling (-) and forcing (+) (no scale), and the x-axis shows calendar time (approximate).

In our conceptual model (Figure 2.2), a few weeks to months of cold temperatures are required to break endodormancy. Subsequently, ecodormancy starts as the chilling requirements to break endodormancy are fulfilled. During the ecodormancy period, forcing above a certain temperature threshold is critical for accumulating heat units, and as buds experience temperatures above the threshold, the ecodormancy is released to reach flowering. Our conceptual models are based on this understanding, and chilling ( $R_c$ ) and forcing requirements ( $R_f$ ) are fixed for the cherry to bloom in our conceptual model (Figure 2.2). If the chilling period is colder than usual (CC),  $R_c$  will be fulfilled earlier (steeper negative slope during chilling period in Figure 2.2a and 2.2b); on the other hand, if it is warmer than usual (WC),  $R_c$  will be fulfilled later (more gradual negative slope during chilling period in Figure 2.2c and 2.2d). Similarly, if the forcing period is warmer (WF), the heat accumulation will reach  $R_f$  faster (steeper positive slope during forcing period in Figure 2.2a and 2.2c). If it is colder than usual (CF), the heat accumulation will reach  $R_f$  later (more gradual positive slope during forcing period in Figure 2.2b and 2.2d). We expect early (Figure 2.2a), medium (Figure 2.2b and 2.2c), and late (Figure 2.2d) bloom dates based on the combination of colder or warmer temperatures during chilling and forcing periods.

### 2.2.3 Analysis of warming effects during chilling and forcing periods

We implemented one-way analysis of variance (ANOVA) and Fisher's Protected Least Significant Difference (LSD) to test the warming effects of chilling and forcing temperatures on observed Yoshino cherry bloom dates in different chilling-forcing temperature combinations and locations and two-way ANOVA to test the main effects of cold or warm chilling and forcing and interaction between these two. We also calculated the deviation from the average bloom

date across all latitudes for each site and analyzed the bloom dates on a temporal scale before and after 1980 because cold chilling and forcing (CC-CF) happened more frequently before 1980, while warm chilling and forcing (WC-WF) happened more regularly after 1980 (Table SA2.2 and Figure SA2.7). We then calculated each location's temperature sensitivity ( $S_T$ ; Fu et al., 2015).  $S_T$  is defined as the advance (-) or delay (+) of full bloom dates per degree Celsius changes of chilling or forcing mean temperature ( $\Delta$  bloom date /  $\Delta$  mean chilling or forcing temperature, unit: day/°C). The calculation of  $S_T$  was based on the comparisons of the following four scenarios: (1) CC-CF and CC-WF years in each location shared similar cold chilling temperatures but different forcing temperatures; (2) WC-CF and WC-WF combinations had similar warm chilling temperatures but different forcing temperatures; (3) CC-CF and WC-CF differed by the chilling temperature but had similar cold forcing temperatures., and (4) CC-WF and WC-WF years had differing chilling temperatures but shared similar warm forcing temperatures.

#### 2.2.4 Calculation of chilling and forcing requirements

After identifying chilling and forcing phases, start and end dates were extracted from the PLS regression results based on the criteria described in Section 2.2.2. For the chilling model, we applied the Dynamic Model (Fishman et al., 1987b, 1987a), which consistently performed the best in most cases (Campoy et al., 2011; Guo et al., 2015a; Guo et al., 2013; Guo et al., 2015b; Luedeling et al., 2009a, 2009b; Luedeling & Gassner, 2012; Ruiz et al., 2007; Zhang & Taylor, 2011). The Dynamic Model hypothesizes that winter chill accumulates in a two-step process. At first, cold temperatures form an intermediate product that could be diminished under high temperatures. Once a certain amount of this intermediate product has

accumulated, it can be converted into a Chill Portion (CP) by a process requiring relatively moderate temperatures. For the forcing model, we followed the Growing Degree Hours Model (GDD; Anderson et al., 1986), a model using a base temperature of 4 °C, an optimum temperature of 25°C, and a critical temperature of 36°C, as suggested by Anderson et al. (1986) for fruit trees. The equations used for calculating chill and heat accumulation are shown in Supplementary materials B (Luedeling et al., 2009a; Luedeling & Brown, 2011). We then used the PLS-defined chilling and forcing periods (Table SA2.1) to calculate Yoshino cherry's chilling and forcing requirements as the total chill and heat units accumulated during these periods. All analyses were conducted in R programming language (version 4.1.2; R Core Team, 2021) with *chillR* package (Luedeling and Fernandez, 2022).

## 2.3 Results

### 2.3.1 Winter and spring warming exerted varying effects on bloom dates across different latitudes, and the change of bloom date is driven by the net effect of chilling and forcing.

Annual mean temperatures, chilling period mean temperatures, and forcing period mean temperatures increased consistently during the past seven decades in all locations along the latitudinal gradient (Figure SA2.1). The Yoshino cherry bloomed earlier correspondingly at all locations except the two lowest-latitude locations, Miyazaki and Kagoshima (Figure SA2.2). The rate of advancement in bloom dates was more prominent in higher latitudes than in lower latitudes (greater slope in higher latitudes in Figures SA2.2). It was also apparent that temperatures were generally lower in higher latitudes than in lower latitudes (Figure SA2.1a), leading to later bloom dates in high-latitude locations (Figure SA2.1d, Tables 2.1, and SA2.4).

However, the bloom dates did not vary substantially among the locations at latitudes lower than Sendai's latitude (38.2°N; Figure SA2.1d, Tables 2.1 and SA2.4), and our analysis showed that the bloom dates did not always advance accordingly in all locations while temperatures continued to rise in the past seven decades (Figures SA2.1 and SA2.2). This reinforces the need to look into the warming effects of chilling and forcing periods separately from both temporal and spatial perspectives.

The two-way ANOVA indicated that warmer forcing temperatures consistently advanced the bloom dates irrespective of chilling temperatures in all locations (forcing temperature  $p \leq 0.001$ ; Table 2.1). The chilling temperature affected the bloom dates significantly at both higher and lower latitude locations in opposing directions. No interaction between these two main effects was found, except for Tokushima ( $p \leq 0.05$ ). Increasing temperatures during chilling periods advanced bloom dates at high latitudes (Sapporo:  $p \leq 0.01$ , Aomori:  $p \leq 0.05$ , Sendai:  $p \leq 0.05$ , Tokyo:  $p \leq 0.05$ ; Table 2.1) but delayed them at low latitudes (Kagoshima and Miyazaki:  $p \leq 0.01$ , Kochi:  $p \leq 0.05$ ; Table 2.1) while no effect was found at mid latitudes. These results highlight that warming during the chilling period had differing effects on bloom dates along the latitudinal gradient. The ANOVA further revealed that the bloom dates were significantly different between combinations of the chilling-forcing temperatures (i.e., CC-CF, CC-WF, WC-CF, and WC-WF) in all locations ( $p \leq 0.001$ ; Table SA2.4). For instance, CC-CF and WC-CF had similar later bloom dates, while CC-WF and WC-WF had similar earlier bloom dates in most of the higher latitudes (i.e., above Fukuoka, except Aomori and Tokushima; Table SA2.4). Interestingly, the earlier and later bloom dates within the above two comparisons changed at lower latitudes. The CC-CF had much earlier bloom dates than WC-CF in the lowest two latitudes: Miyazaki and Kagoshima (Table SA2.4). Similarly, the WC-WF combinations had

significantly later bloom dates than the CC-WF combinations in most low-latitude locations below 35°N (Table SA2.4). This result indicates that bloom dates were delayed at lower latitudes when the chilling period was warmer than usual despite a warmer forcing period characterized as a warm spring.

Table 2.1 Average bloom dates and standard errors of Yoshino cherry of all years and deviation from the average bloom date of all years in different chilling and forcing temperatures (CC: cold chilling; WC: warm chilling; CF: cold forcing; WF: warm forcing), and two-way ANOVA results of chilling and forcing temperatures in different locations. The interaction term of the main effects is non-significant, except for Tokushima  $p < 0.05$ .

Location	Latitude (°N)	Average bloom date of all years	Deviation from the average bloom date of all years					
			CC	WC	Chilling temperature $p$ -value	CF	WF	Forcing temperature $p$ -value
Sapporo	43.06	127.7 ± 0.7	1.5 ± 0.8	-1.6 ± 1.0	<0.01 **	3.6 ± 0.7	-3.7 ± 0.8	≤0.001 ***
Aomori	40.82	119.7 ± 0.7	1.3 ± 1.1	-1.4 ± 0.9	<0.05 *	4.2 ± 0.7	-4.4 ± 0.8	≤0.001 ***
Sendai	38.26	106.1 ± 0.7	1.2 ± 1.0	-1.2 ± 0.9	<0.05 *	3.8 ± 0.7	-3.9 ± 0.8	≤0.001 ***
Tokyo	35.69	93.6 ± 0.7	1.0 ± 1.0	-1.0 ± 0.9	<0.05 *	3.8 ± 0.6	-3.9 ± 0.8	≤0.001 ***
Yokohama	35.44	94.0 ± 0.6	0.6 ± 0.8	-0.6 ± 0.9	0.229	2.9 ± 0.7	-3.0 ± 0.7	≤0.001 ***
Kyoto	35.01	96.1 ± 0.5	0.7 ± 0.8	-0.7 ± 0.6	0.120	2.5 ± 0.6	-2.6 ± 0.6	≤0.001 ***
Tokushima	34.07	95.4 ± 0.4	-0.4 ± 0.7	0.5 ± 0.5	0.154	2.1 ± 0.4	-2.2 ± 0.6	≤0.001 ***
Fukuoka	33.58	92.5 ± 0.6	0.7 ± 0.8	-0.8 ± 0.8	0.102	2.8 ± 0.5	-2.9 ± 0.7	≤0.001 ***
Kochi	33.57	90.0 ± 0.6	-1.1 ± 0.8	1.2 ± 0.8	<0.05 *	2.3 ± 0.7	-2.4 ± 0.8	≤0.001 ***
Oita	33.24	94.7 ± 0.5	-0.2 ± 0.7	0.2 ± 0.8	0.569	2.3 ± 0.5	-2.3 ± 0.7	≤0.001 ***
Miyazaki	31.94	92.5 ± 0.5	-1.3 ± 0.6	1.3 ± 0.7	<0.01 **	2.0 ± 0.6	-2.1 ± 0.7	≤0.001 ***
Kagoshima	31.56	94.0 ± 0.6	-1.6 ± 0.6	1.7 ± 0.9	<0.01 **	1.0 ± 0.7	-1.1 ± 0.9	≤0.001 ***

\*:  $p \leq 0.05$ ; \*\*:  $p \leq 0.01$ ; \*\*\*:  $p \leq 0.001$

As expected, CF and WF consistently delayed (+) and advanced (-) bloom dates, respectively (Table 2.1, Figure 2.3b, 2.3d, SA2.3b, and SA2.3d) in all locations. The absolute deviations from the average bloom dates in CF and WF increased with increasing latitudes (Figure 2.3b, 2.3d, SA2.3b, and SA2.3d). On the contrary, chilling temperatures had differential effects along the latitudinal gradient. CC delayed bloom dates at higher latitudes and advanced bloom dates at lower latitudes, while WC had an opposite trend of advanced bloom dates at higher latitudes and delayed bloom dates at lower latitudes (Figures 2.3a, 2.3c, SA2.3a, and SA2.3c). The inflection points between the advance and delay of bloom dates in both CC and WC were about 35°N (Figure 2.3a and 2.3c). The deviation from the average bloom dates in four chilling-forcing combinations along the latitudinal gradient revealed more details about the counteracting and additive effects of chilling and forcing temperatures. The CC-WF and WC-CF combinations advanced and delayed the bloom dates, respectively, in all locations with no exceptions (Figures SA2.4b and SA2.4c) because CF and WF dominated counteracting effects of WC and CC at higher latitudes and had additive effects on WC and CC at lower latitudes in the CC-WF and WC-CF combinations, resulting in no latitudinal gradient in bloom dates (Figure SA2.5b and SA2.5c). However, CC-CF and WC-WF combinations did not display a consistent pattern across the latitudinal gradient (Figure SA2.4a and SA2.4d). Overall, the warmer forcing temperature consistently advanced the bloom dates along the latitudinal gradient, while the warmer chilling temperature could advance, delay the bloom dates, or have no effect, depending on the latitudes. The forcing temperature has been the primary driver of changes in bloom dates, but the chilling temperature has begun to emerge as a strong secondary driver in high and low latitude locations in opposing directions.

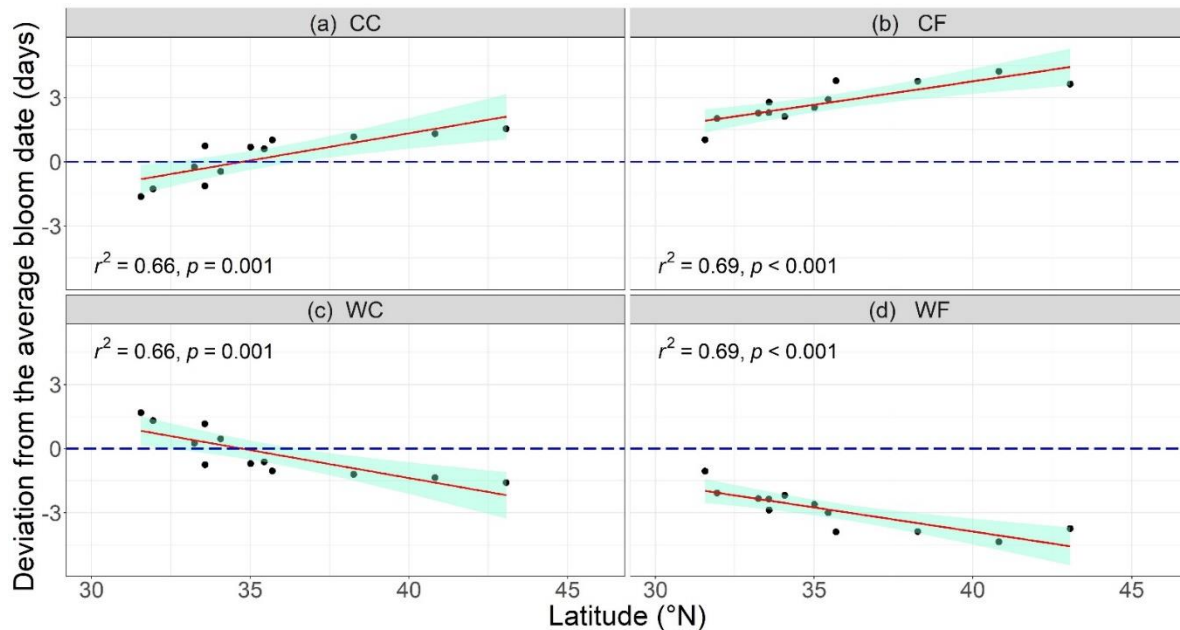


Figure 2.3 A simple linear regression of the deviation of Yoshino cherry’s bloom dates from the average bloom dates in different chilling and forcing temperatures across the latitudinal gradient over the entire study period from 1953 to 2021. (a) CC: cold chilling; (b) CF: cold forcing; (c) WC: warm chilling; (d) WF: warm forcing. Blue shadows are 95% confidence intervals.

### 2.3.2 Earlier spring events caused by spring warming could be offset or overridden by strong winter warming.

In the comparison of “CC-CF & CC-WF” and “WC-CF & WC-WF”, we found that  $S_T$  for forcing temperatures advanced bloom dates at all latitudes as expected and ranged from -1.61 to -6.48 day/°C after cold chilling (CC) and from -2.11 to -5.57 day/°C after warm chilling (WC) periods (Figure 2.4a and 2.4b; Table SA2.5). In the comparison of “CC-CF & WC-CF” and “CC-WF & WC-WF”, the values of  $S_T$  for chilling were mostly positive, indicating a degree increase in

chilling temperature delayed bloom dates at most latitudes with an exception in Sapporo. The  $S_T$  ranged from -0.01 to 2.88 day/°C followed by cold forcing (CF) and -0.7 to 2.92 day/°C followed by warm forcing (WF) periods (Figure 2.4c and 2.4d; Table SA2.5). In general,  $S_T$  contributed by forcing temperatures advanced the bloom dates with different magnitudes along the altitudes, while  $S_T$  contributed by chilling temperatures had differential effects along the latitudes. In addition, the magnitude of  $S_T$  in the forcing period was more significant than that in the chilling period, with an exception where  $S_T$  in the chilling period was slightly stronger than  $S_T$  in the forcing period in lower latitude locations: Kagoshima and Miyazaki (Figure 2.4, SA2.6, and Table SA2.5), resulting in the delay of bloom dates that have begun manifesting at lower latitudes (Figure SA2.4a and SA2.4d). In other words, earlier spring events caused by spring warming could be offset or overridden by strong winter warming in these locations.

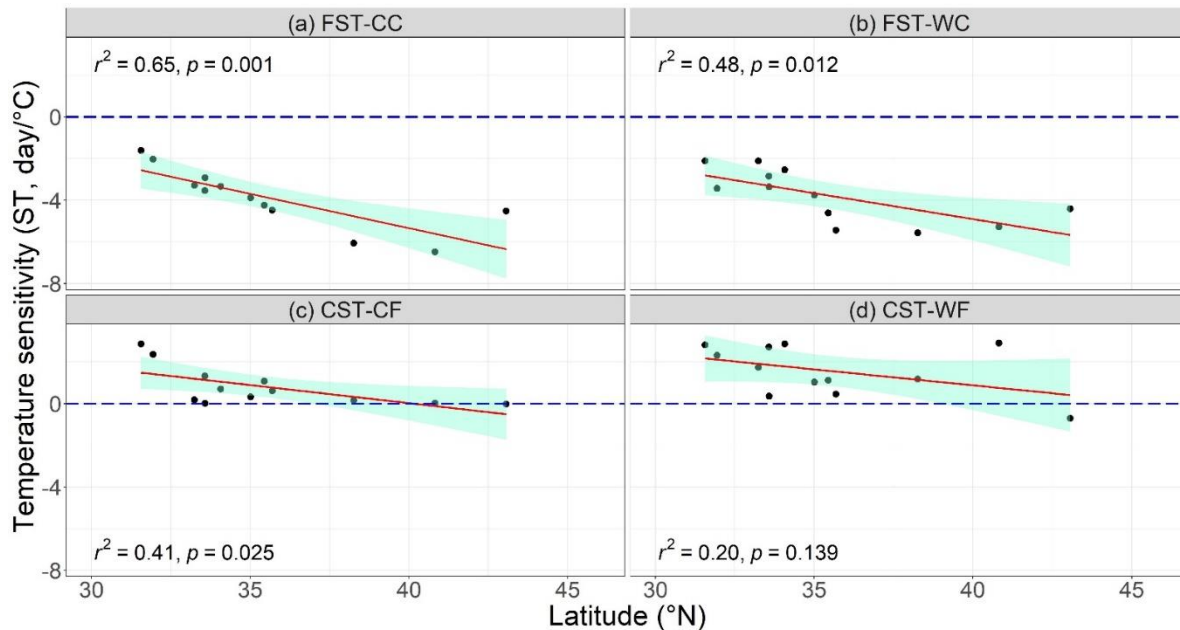


Figure 2.4 A simple linear regression of temperature sensitivity ( $S_T$ , day/°C) over the entire study period from 1953 to 2021 across the latitudinal gradient.  $S_T$  was calculated based on the comparisons described in the materials and methods. (a) FST-CC,  $S_T$  in forcing period after cold chilling temperature; (b) FST-WC,  $S_T$  in forcing period after warm chilling temperature; (c) CST-CF,  $S_T$  in chilling period followed by cold forcing temperature; (d) CST-WF,  $S_T$  in chilling period followed by warm forcing temperature.

### 2.3.3 Flowering time could delay rather than advance under warming, and this has emerged at the lowest latitudes.

Years of cold chilling and cold forcing (CC-CF) were primarily identified before 1980, while warm chilling and warm forcing (WC-WF) were found mainly after 1980 (Figure SA2.7 and Table SA2.2). Our analysis across these two combinations showed that no advancing trends were found in CC-CF in all locations except Kagoshima 31.56°N ( $p \leq 0.001$ ; Figure SA2.8). On the

contrary, most high-latitude locations, i.e., Aomori, Sendai, Tokyo, and Kyoto, had advancing trends in WC-WF ( $p \leq 0.05$ ; Figure SA2.8), while mid to low-latitude locations did not show clear trends, but southernmost locations (i.e., in Oita, Miyazaki, and Kagoshima) exhibited delayed bloom dates (positive slopes in Figure SA2.8). When analyzed separately before and after 1980, the bloom date in Kagoshima did not change before 1980 but began to delay after 1980 ( $p=0.097$ ; Figure SA2.9 and SA2.10). Our results illustrate that the rate at which the bloom date changes differs by location to exhibit a latitudinal gradient (Figure 2.5), and the rate of change across latitudes is significantly different before and after 1980 ( $p<0.001$ ). The interaction between the periods and latitudes is also significant ( $p<0.01$ ).

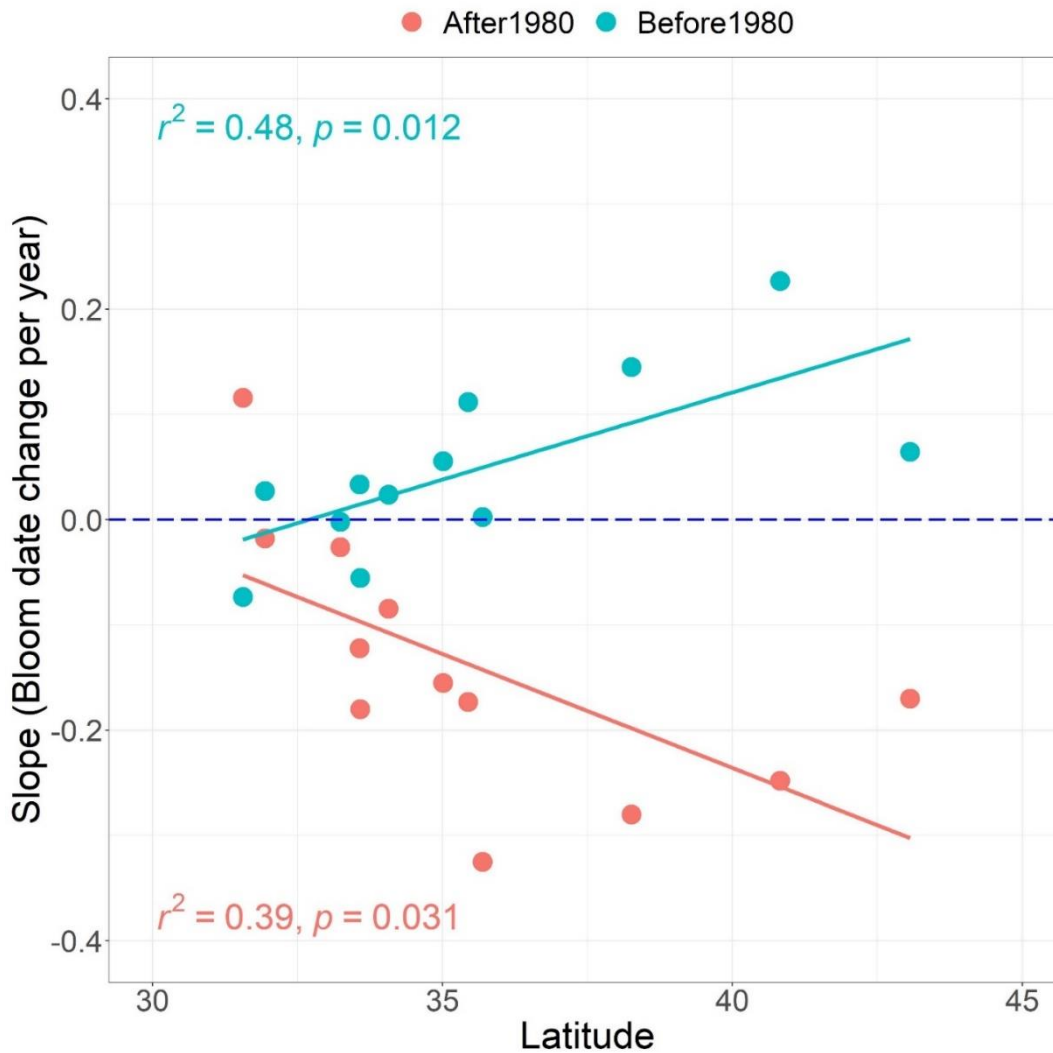


Figure 2.5 A simple linear regression of the bloom date change per year before 1980 (slope before 1980 in Figure SA9) and after 1980 (slopes after 1980 in Figure SA9) across the latitudinal gradient. Positive slope: delay of the bloom date; Negative slope: advance of the bloom date. Change rates of slope across latitudes are significantly different before and after 1980 ( $p < 0.001$ ). The interaction between the periods and latitudes is also significant ( $p < 0.01$ ).

## 2.4 Discussion

### 2.4.1 Warming in chilling and forcing periods affected Yoshino cherry bloom dates differentially across spatial and temporal scales.

Although bloom date did not necessarily advance every year, we observed an advancing trend in most locations in the past seven decades. The results from annual mean temperatures, chilling period mean temperatures, and forcing period mean temperatures and their relations to bloom dates show that temperature drives the Yoshino cherry's bloom dates. However, the average bloom dates do not vary significantly in locations lower than Sendai's latitude ( $38.26^{\circ}\text{N}$ ). This result signifies that the magnitude of the warming effect on flowering time was different between northern and southern latitudes in relation to Sendai.

Our results confirm that warmer forcing temperatures consistently advanced the bloom dates along the latitudinal gradient. However, warmer chilling temperatures advanced bloom dates at higher latitudes, delayed bloom dates at lower latitudes, and had no effect at mid latitudes. The additive and counteracting effects of different chilling- and forcing- temperature combinations along the latitudinal gradient could result in divergent effects of an advance or delay in bloom dates depending on locations. While the effect of forcing temperatures dominated the observed changes in bloom dates, this dominance dissolved with decreasing latitudes. Additionally, warm temperatures during the chilling period likely contributed to slow bloom processes at lower latitudes. Combined together, these effects led to a delay in bloom date at the lowest latitude, Kagoshima, which mostly experienced a warm chilling and forcing combination (WC-WF). The frequency of warm chilling and warm forcing (WC-WF) is likely to intensify and expand to higher latitudes in the future climate, making the delay of bloom dates more frequent and broader in range. These results are consistent with the observations that

forcing plays a major role at higher latitudes, whereas chilling is the main driver at lower latitudes (Guo et al., 2015a). This highlights the need to consider the net balance between these antagonistic warming effects on spring phenology (Chuine et al., 2010) when running phenology analyses over large spatial scales (Wenden et al., 2020).

Our results support the hypothesis proposed by Fu et al. (2015) that earlier spring events caused by spring warming could be compensated by warmer winters, leading to a slowdown of spring event advancement in recent years. Furthermore, we find that this slowdown of spring phenology advancement happened not only on the temporal scale but also on the spatial scale, such that the slowdown was only observed at lower latitudes. Asse et al. (2018) also found that warmer winters reduce the advancement of tree spring phenology along an elevation gradient in the Alps. Warmer chilling periods immediately delay spring phenology in lower latitudes because the inherent warmer chilling temperatures in these regions take longer to meet the chilling requirements of Yoshino cherry due to recent warming. In contrast, at higher latitudes, the chilling period temperature is lower than the optimal chilling temperature, and consequently, when the chilling period temperature rises, sufficient chilling units are accumulated to release endodormancy. The mid latitudes are likely in the transition phase where no effects are observable yet. If temperatures during the chilling period keep climbing in locations at mid to high latitudes, a delay in bloom dates will likely be observed eventually. Some studies similarly suggest that the accumulation of chilling units is consistently reduced in warm regions with winter warming, while winters are usually too cold for optimal chilling in temperate regions, and winter warming can actually shorten the time required for chilling accumulation (Asse et al., 2018; Ettinger et al., 2020; Güsewell et al., 2017; Kaufmann & Blanke, 2019; Luedeling, 2012; Luedeling et al., 2011). This implies that chilling units in winter are

currently sufficient to meet the chilling requirements in high latitudes, but this may not be the case under future climate change scenarios (Cook et al., 2012b; Yu et al., 2010). Therefore, a reliable chilling model is critical to assessing the spring phenology of temperate trees in long-term projections (Yun et al., 2017).

#### 2.4.2 Temperature sensitivity ( $S_T$ ) of chilling and forcing periods on Yoshino cherry bloom dates across a latitudinal gradient

The magnitude of  $S_T$  in the forcing period was more significant than that in the chilling period when considering all locations. However,  $S_T$  in the chilling period was slightly stronger than  $S_T$  in the forcing period at lower latitudes (Figure 2.5).  $S_T$  provides quantified values showing that these counteracting effects resulting in a delay of bloom dates due to winter warming are observed in recent records at low-latitude locations in Japan. Fu et al., (2015) found that all species in their study show a similar significant decrease in  $S_T$  over time. Their results suggest winter warming as the driver for reduced leaf unfolding responsiveness to ongoing climate warming in all studied tree species in Central Europe. We find the same phenomenon happened for cherry bloom dates along the latitudinal gradient. While the present phenomenon only manifests in locations at lower latitudes, it may extend to higher latitudes in the near future if the climate continues to change rapidly. In contrast, Fu et al. (2015) did not observe changes in  $S_T$  for spring leaf unfolding across the latitudinal gradient, neither across all species nor for individual species in their study. This may be due to their study sites spanning from the Adriatic Sea to north Germany (43°N to 57°N) with higher latitudes than our sites (ranging from 31°N to 43°N). Lastly, our focus on reproductive phenology may be

another reason for the discrepancy in ST responses observed in our results compared to Fu et al. (2015), which focused on vegetative phenology.

Both phenotypic plasticity and adaptive evolution can contribute to phenological timing shifts in response to climate change (Anderson et al., 2012). Adaptation is relatively slow for long-lived species, such as trees, so changes in flowering time are likely primarily due to phenotypic plasticity (Anderson et al., 2012; Franks et al., 2014). Iketani et al. (2007) analyzed the clonal status of Yoshino cherries planted from the late 19th to the early 20th century from various locations in Japan. They found that 50 out of 52 individuals showed an identical genotype. Likely, Yoshino cherries in our sites are genetically identical, which means the different bloom dates at different locations are contributed by the phenotypic plasticity related to environmental acclimation. Therefore, differences in bloom dates between different locations can be attributed to phenotypic plasticity in response to localized environmental trends rather than recent genetic adaptation. The changes in average bloom dates from 128 (Sapporo) to 90 (Kochi) days of the year (Table 2.1) demonstrate the acclimation potential of flowering time for the Yoshino cherry across the latitudinal gradient under the current climate. Nevertheless, it is unclear whether phenotypic plasticity can totally accommodate future temperature changes (Richardson et al., 2017) that create unpredictability in spring phenology. A study of apple and pear trees showed no bloom under warm conditions (between 12 and 18 °C in both chilling and forcing periods) (Fernandez et al., 2021). Likely, Yoshino cherry will soon reach a similar ceiling temperature where it may not flower. Therefore, understanding the variation of phenotypic plasticity across a spatial gradient is useful for improving model predictions in the future.

### 2.4.3 Chilling and forcing requirements for Yoshino cherry to bloom across the latitudinal gradient.

The reverse trends of chilling and forcing requirements along the latitudinal gradient show that in locations with lower chilling requirements, Yoshino cherry also requires more forcing to bloom (Tables SB2.1, SB2.3, Figures SB2.4, and SB2.8), consistent with (Harrington et al., 2010; Harrington & Gould, 2015; Kaufmann & Blanke, 2019; Luedeling et al., 2013b; Okie & Blackburn, 2011). However, it is notable that chilling requirements should be significantly different between cold and warm chilling combinations. Cold or warm chilling temperatures do not affect the chilling requirements at higher latitude locations (Table SB2.2). We applied the Dynamic Model (Fishman et al., 1987b, 1987a) using a parameter set that has been widely used in fruit trees in recent years (Campoy et al., 2011; Guo et al., 2015a; Guo et al., 2013; Guo et al., 2015b; Luedeling et al., 2009a, 2009b; Luedeling & Gassner, 2012; Ruiz et al., 2007; Zhang & Taylor, 2011) to calculate chilling requirements. In this approach, a hypothetical intermediate product is first formed, which is vulnerable to destruction by warm temperatures. Another possibility is that the effects of cold temperatures are not adequately described in the model or chill accumulation rates are homogeneous across a broader range of temperatures, contrary to what Dynamic Model assumes (Luedeling et al., 2013b), which means the parameter set is not ideal for Yoshino cherry in Japan.

Similarly, chilling temperatures should not affect forcing requirements between cold and warm forcing combinations. However, warm chilling temperatures lead to greater forcing requirements at most latitudes (Table SB2.4) due to the overlap of the chilling and forcing periods (Table SA2.1). When chilling temperatures are warmer, the forcing temperatures are also warmer in the overlapping period resulting in higher forcing requirements. Our results of

the deviation from the average chill portion (CP) and growing degree days (GDD) (Figures SB2.1 to SB2.8) could not completely explain bloom date changes along the latitudinal gradient (Figures SA2.4 and SA2.5) but forcing requirements explain more than chilling requirements. It is possible that the unexplained part of the bloom date change is concealed in the warming trends of the past decades, implying that the chilling and forcing periods may have also changed during the past decades (Wenden et al., 2020). We might be able to explore this option with different periods of data by using PLS to detect the chilling and forcing periods to shift over time with increasing observations in the future. Therefore, it is worth disentangling the relationship between warming trends and changes in chilling and forcing periods over time in the future.

#### 2.4.4 Study limitations

We found the bloom date in Kagoshima did not change before 1980 but began to delay after 1980 ( $p=0.097$ ). It showed that the statistical evidence of a delay in bloom date is weak, and this is likely because the signal is weak and not easy to detect by the procedure we used in our paper. However, with increasing observations in the future or other long-term data from lower latitudes than Kagoshima ( $31.56^{\circ}\text{N}$ ), we can likely see a more substantial delaying effect. We define chilling and forcing periods by using PLS regression (Luedeling & Gassner, 2012; Wenden et al., 2020) to analyze the influence of daily temperatures on cherry bloom dates. However, the criteria to define the start and end days are subjective unless using a method similar to the approach used by (Wenden et al., 2020). Though the variable importance (VIP) and coefficients of PLS regression were defined in the past (Luedeling & Gassner, 2012), PLS results are not always unambiguous (Luedeling et al., 2013a). Therefore, Wenden et al. (2020)

suggested using the same VIP and coefficient criteria as Luedeling & Gassner (2012), plus the consecutive days that coefficients were above or below the criteria to make it more consistent to start with.

Still, the PLS regression does not always work, especially when it comes to the chilling period (Luedeling et al., 2013a); for example, the identification of start and end dates of the chilling period at Sendai in our study. We also visualized the PLS-generated patterns to inspect the chilling and forcing periods with expert judgment. One possible reason for lower PLS regression performance may be that forcing is currently a much stronger driver in spring phenology than chilling (Luedeling et al., 2013a) at higher latitudes. It is also possible that the clarity of the chilling pattern in the PLS results is related to the chilling temperature range at the study site (Luedeling et al., 2013a) because analysis of phenological records by PLS requires a certain level of input data variability. If the independent variables do not vary sufficiently, the PLS cannot detect responses to variation. Another possibility is having a relatively small dataset containing high variation (Luedeling et al., 2013a). PLS analysis does well in a situation with many observations and a clear normal baseline temperature pattern. If temperatures vary highly, it is difficult to create a normal pattern. Though the use of PLS regression is a departure from more traditional approaches and requires specific criteria to be met, it can more objectively identify the start and end dates of chilling and forcing phases (Wenden et al., 2020). However, long-term datasets are necessary because temperature and phenology variability are required for PLS analysis. We have seven decades of data, but after classifying these data into four different combinations, each combination's sample size became relatively small compared to the whole dataset. In addition, chilling and forcing periods may change from year to year, complicating the phenological temperature sensitivity estimation.

Despite these limitations, the application of PLS regression in this context is well supported by recent literature (Benmoussa et al., 2017; Fernandez et al., 2021; Guo et al., 2015a; Guo et al., 2013; Guo et al., 2015b; Luedeling, 2012; Luedeling et al., 2013b, 2013a; Luedeling & Gassner, 2012; Martínez-Lüscher et al., 2017; Wenden et al., 2020). We addressed the aforementioned limitations by combining PLS with a sound ecological theory guiding us to interpret the results, visualizing PLS-generated patterns, and including as much data as possible. Results are biologically sound, consistent with other studies, and imply potential testable hypotheses for further investigation.

## 2.5 Conclusions

Our study illustrates that warming during chilling and forcing periods shifted bloom dates of Yoshino cherries differentially and exerted varying effects on bloom time across different latitudes in Japan. Forcing temperatures consistently advanced bloom dates irrespective of chilling temperatures at all locations. In contrast, the chilling temperatures significantly affected the bloom dates at higher and lower latitude locations in different directions. That is, advancing at high latitudes and delaying at low latitudes. Our findings support the slowdown of phenological shifts caused by warmer winters in recent years. The temperature sensitivities of chilling and forcing periods in our study provide quantitative evidence showing that these counteracting effects in spring phenology due to warming during the chilling period are observed in recent records from low-latitude locations in Japan. These findings highlight that the flowering time could delay rather than advance under temperature warming. This phenomenon has begun to emerge in low latitude locations and may extend to higher latitudes in the future.

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Supplementary materials A

Table SA2.1. The estimation of start and end dates of chilling and forcing periods of each location over the entire study period from 1953 to 2021 by using Partial Least Squares (PLS) regression.

Location	Chilling period				Forcing period			
	Start		End		Start		End	
	Day of year	Date	Day of year	Date	Day of year	Date	Day of year	Date
Sapporo	255	Sep 11	33	Feb 2	42	Feb 11	128	May 8
Aomori	267	Sep 23	3	Jan 3	360	Dec 25	120	Apr 30
Sendai	296	Oct 22	4	Jan 4	352	Dec 17	107	Apr 17
Tokyo	288	Oct 14	28	Jan 28	30	Jan 30	94	Apr 4
Yokohama	276	Oct 2	5	Jan 5	6	Jan 6	94	Apr 4
Kyoto	315	Nov 10	33	Feb 2	13	Jan 13	97	Apr 7
Tokushima	263	Sep 19	6	Jan 6	50	Feb 19	96	Apr 6
Fukuoka	254	Sep 10	6	Jan 6	33	Oct 11	93	Apr 3
Kochi	301	Oct 27	7	Jan 7	16	Jan 16	90	Mar 31
Oita	269	Sep 25	6	Jan 6	51	Feb 20	95	Apr 5
Miyazaki	267	Sep 23	12	Jan 12	32	Feb 1	93	Apr 3
Kagoshima	249	Sep 5	13	Jan 13	49	Feb 18	94	Apr 4

Table SA2.2. Years were classified as different combinations: cold chilling-cold forcing (CC-CF), cold chilling-warm forcing (CC-WF), warm chilling-cold forcing (WC-CF), and warm chilling-warm forcing (WC-WF) in in each location. The numbers in the parentheses are the numbers of years in each combination.

Location	Latitude	Years of CC-CF	Years of CC-WF	Years of WC-CF	Years of WC-WF
Sapporo	43.06	1953, 1954, 1955, 1956, 1957 1960, 1961, 1962, 1965, 1966 1969, 1970, 1971, 1974, 1975 1976, 1977, 1978, 1979, 1981 1984, 1986, 1987, 1988 <b>(24)</b>	1959, 1963, 1967, 1968, 1972 1982, 1985, 2001, 2003, 2017 2021 <b>(11)</b>	1958, 1964, 1973, 1980, 1996 1999, 2000, 2005, 2010, 2012 2013 <b>(11)</b>	1983, 1989, 1990, 1991, 1992 1993, 1994, 1995, 1997, 1998 2002, 2004, 2006, 2007, 2008 2009, 2011, 2014, 2015, 2016 2018, 2019, 2020 <b>(23)</b>
Aomori	40.82	1953, 1954, 1955, 1957, 1960 1961, 1964, 1965, 1966, 1967 1968, 1969, 1970, 1971, 1974 1975, 1976, 1977, 1982, 1984 1985, 1986, 1987, 1988, 2001 2006 <b>(26)</b>	1959, 1963, 1972, 1989, 2002 2003, 2017, 2018, 2021 <b>(9)</b>	1956, 1958, 1978, 1980, 1981 1996, 2005, 2012, 2013 <b>(9)</b>	1962, 1973, 1979, 1983, 1990 1991, 1992, 1993, 1994, 1995 1997, 1998, 1999, 2000, 2004 2007, 2008, 2009, 2010, 2011 2014, 2015, 2016, 2019, 2020 <b>(25)</b>
Sendai	38.26	1953, 1955, 1956, 1957, 1960 1961, 1963, 1965, 1967, 1968 1970, 1971, 1974, 1975, 1976 1977, 1981, 1984, 1985, 1986 1987, 1996, 2001, 2006, 2013 <b>(25)</b>	1954, 1959, 1966, 1972, 1982 1989, 2002, 2003, 2015, 2018 <b>(10)</b>	1958, 1962, 1964, 1969, 1978 1980, 1988, 2005, 2011, 2012 <b>(10)</b>	1973, 1979, 1983, 1990, 1991 1992, 1993, 1994, 1995, 1997 1998, 1999, 2000, 2004, 2007 2008, 2009, 2010, 2014, 2016 2017, 2019, 2020, 2021 <b>(24)</b>
Tokyo	35.69	1953, 1954, 1955, 1956, 1957 1958, 1961, 1962, 1963, 1964 1965, 1967, 1968, 1970, 1971 1972, 1974, 1975, 1977, 1981 1984, 1985, 1986, 2003 <b>(24)</b>	1959, 1960, 1966, 1976, 1982 1987, 2006, 2013, 2017, 2018 2021 <b>(11)</b>	1969, 1973, 1978, 1980, 1983 1988, 1994, 1995, 1996, 2011 2012 <b>(11)</b>	1979, 1989, 1990, 1991, 1992 1993, 1997, 1998, 1999, 2000 2001, 2002, 2004, 2005, 2007 2008, 2009, 2010, 2014, 2015 2016, 2019, 2020 <b>(23)</b>
Yokohama	35.44	1953, 1954, 1955, 1956, 1957 1958, 1959, 1961, 1962, 1963 1964, 1965, 1967, 1968, 1970 1971, 1974, 1975, 1976, 1977 1981, 1984, 1985, 1986, 2003 <b>(25)</b>	1960, 1966, 1972, 1979, 1982 1987, 1989, 2006, 2013, 2018 <b>(10)</b>	1969, 1978, 1980, 1983, 1988 1994, 1996, 2001, 2011, 2012 <b>(10)</b>	1973, 1990, 1991, 1992, 1993 1995, 1997, 1998, 1999, 2000 2002, 2004, 2005, 2007, 2008 2009, 2010, 2014, 2015, 2016 2017, 2019, 2020, 2021 <b>(24)</b>

Kyoto	35.01	1953, 1956, 1957, 1960, 1961 1962, 1963, 1965, 1966, 1967 1968, 1970, 1971, 1974, 1975 1976, 1977, 1981, 1982, 1984 1986, 1996, 2003, 2011, 2012 <b>(25)</b>	1954, 1955, 1959, 1985, 1990 2006, 2013, 2014, 2015, 2018 <b>(10)</b>	1958, 1964, 1969, 1978, 1980 1983, 1988, 1994, 2000, 2008 <b>(10)</b>	1972, 1973, 1979, 1987, 1989 1991, 1992, 1993, 1995, 1997 1998, 1999, 2001, 2002, 2004 2005, 2007, 2009, 2010, 2016 2017, 2019, 2020, 2021 <b>(24)</b>
Tokushima	34.07	1953, 1956, 1957, 1963, 1964 1965, 1968, 1969, 1970, 1971 1972, 1973, 1974, 1975, 1976 1981, 1984, 1985, 1986, 1987 1996, 2003, 2006 <b>(23)</b>	1955, 1958, 1959, 1966, 1967 1977, 1982, 1989, 2002, 2013 2018, 2021 <b>(12)</b>	1962, 1978, 1980, 1983, 1988 1991, 1993, 1994, 2000, 2005 2011, 2012 <b>(12)</b>	1954, 1960, 1961, 1979, 1990 1992, 1995, 1997, 1998, 1999 2001, 2004, 2007, 2008, 2009 2010, 2014, 2015, 2016, 2017 2019, 2020 <b>(22)</b>
Fukuoka	33.58	1953, 1954, 1956, 1957, 1958 1961, 1963, 1964, 1965, 1967 1968, 1969, 1970, 1971, 1972 1974, 1975, 1977, 1980, 1981 1984, 1986, 1996 <b>(23)</b>	1955, 1959, 1966, 1973, 1976 1982, 1987, 1989, 2003, 2006 2013, 2021 <b>(12)</b>	1962, 1978, 1983, 1985, 1988 1991, 1994, 2000, 2005, 2008 2011, 2012 <b>(12)</b>	1960, 1979, 1990, 1992, 1993 1995, 1997, 1998, 1999, 2001 2002, 2004, 2007, 2009, 2010 2014, 2015, 2016, 2017, 2018 2019, 2020 <b>(22)</b>
Kochi	33.57	1956, 1957, 1958, 1961, 1962 1963, 1964, 1965, 1967, 1968 1970, 1971, 1974, 1975, 1977 1981, 1982, 1984, 1986, 1987 1996 <b>(21)</b>	1955, 1966, 1972, 1973, 1976 1989, 1997, 2002, 2003, 2006 2013, 2014, 2015, 2018 <b>(14)</b>	1953, 1960, 1978, 1980, 1983 1985, 1988, 1993, 1994, 1995 2000, 2005, 2011, 2012 <b>(14)</b>	1954, 1959, 1969, 1979, 1990 1991, 1992, 1998, 1999, 2001 2004, 2007, 2008, 2009, 2010 2016, 2017, 2019, 2020, 2021 <b>(20)</b>
Oita	33.24	1953, 1956, 1957, 1958, 1963 1964, 1965, 1968, 1969, 1970 1971, 1972, 1974, 1975, 1980 1981, 1984, 1986, 1987, 1994 1996 <b>(21)</b>	1955, 1959, 1961, 1966, 1967 1973, 1977, 1982, 1989, 1997 2003, 2006, 2013, 2018 <b>(14)</b>	1954, 1960, 1962, 1976, 1978 1983, 1985, 1988, 1991, 1993 1995, 2000, 2005, 2011 <b>(14)</b>	1979, 1990, 1992, 1998, 1999 2001, 2002, 2004, 2007, 2008 2009, 2010, 2012, 2014, 2015 2016, 2017, 2019, 2020, 2021 <b>(20)</b>
Miyazaki	31.94	1953, 1956, 1957, 1958, 1961 1963, 1964, 1965, 1967, 1968 1970, 1971, 1974, 1975, 1977 1980, 1981, 1984, 1986, 1996 <b>(20)</b>	1955, 1959, 1966, 1972, 1973 1976, 1982, 1987, 1989, 1990 2003, 2006, 2013, 2018, 2021 <b>(15)</b>	1954, 1962, 1969, 1978, 1983 1988, 1993, 1994, 1995, 2000 2005, 2008, 2011, 2012, 2017 <b>(15)</b>	1960, 1979, 1985, 1991, 1992 1997, 1998, 1999, 2001, 2002 2004, 2007, 2009, 2010, 2014 2015, 2016, 2019, 2020 <b>(19)</b>
Kagoshima	31.56	1953, 1954, 1956, 1957, 1958 1961, 1962, 1963, 1964, 1965 1967, 1968, 1969, 1970, 1971 1972, 1973, 1974, 1980, 1981 1984, 1986, 1987, 1996 <b>(24)</b>	1955, 1959, 1960, 1966, 1976 1977, 1982, 1989, 2003, 2006 2021 <b>(11)</b>	1975, 1978, 1983, 1988, 1991 1993, 1994, 1995, 2000, 2005 2017 <b>(11)</b>	1979, 1985, 1990, 1992, 1997 1998, 1999, 2001, 2002, 2004 2007, 2008, 2009, 2010, 2011 2012, 2013, 2014, 2015, 2016 2018, 2019, 2020 <b>(23)</b>

Table SA2.3. Chilling and forcing period mean temperature (°C) and standard deviation of each combination (CC-CF: cold chilling-cold forcing period, CC-WF: cold chilling-warm forcing period, WC-CF: warm chilling-cold forcing period, WC-WF: warm chilling-warm forcing period) and of all years in each location along the latitudinal gradient in Japan from 1953 to 2021.

Location	Latitude (°N)	CC-CF		CC-WF		WC-CF		WC-WF		Chilling period mean temperature of all years (°C)	Forcing period mean temperature of all years (°C)
		mean temperature (°C)	mean temperature (°C)	mean temperature (°C)	mean temperature (°C)	mean temperature (°C)	mean temperature (°C)				
		Chilling	Forcing	Chilling	Forcing	Chilling	Forcing	Chilling	Forcing		
Sapporo	43.06	3.62 ± 0.59	1.88 ± 0.56	3.67 ± 0.38	3.35 ± 0.67	5.06 ± 0.38	2.10 ± 0.58	5.20 ± 0.51	3.84 ± 0.81	4.38 ± 0.91	2.80 ± 1.11
Aomori	40.82	6.42 ± 0.53	0.95 ± 0.54	6.79 ± 0.35	2.70 ± 0.56	7.85 ± 0.47	1.11 ± 0.37	8.06 ± 0.50	2.56 ± 0.55	7.25 ± 0.90	1.78 ± 0.96
Sendai	38.26	6.35 ± 0.58	2.85 ± 0.58	6.47 ± 0.48	4.32 ± 0.59	7.87 ± 0.55	3.16 ± 0.42	8.00 ± 0.46	4.47 ± 0.52	7.16 ± 0.95	3.67 ± 0.92
Tokyo	35.69	9.30 ± 0.42	6.85 ± 0.77	9.60 ± 0.47	8.60 ± 0.92	10.92 ± 0.30	7.33 ± 0.41	11.00 ± 0.42	8.84 ± 0.66	10.17 ± 0.90	7.87 ± 1.14
Yokohama	35.44	11.46 ± 0.39	6.14 ± 0.70	11.88 ± 0.25	7.68 ± 0.53	12.90 ± 0.43	6.64 ± 0.32	13.23 ± 0.48	8.07 ± 0.78	12.35 ± 0.90	7.11 ± 1.09
Kyoto	35.01	5.88 ± 0.56	5.60 ± 0.68	6.35 ± 0.40	7.15 ± 0.52	7.35 ± 0.32	6.08 ± 0.29	7.72 ± 0.53	7.44 ± 0.74	6.80 ± 0.96	6.53 ± 1.04
Tokushima	34.07	13.24 ± 0.41	8.10 ± 0.74	13.26 ± 0.42	10.06 ± 0.88	14.57 ± 0.31	8.67 ± 0.33	14.64 ± 0.58	10.05 ± 0.54	13.92 ± 0.82	9.16 ± 1.11
Fukuoka	33.58	14.03 ± 0.53	7.70 ± 0.70	14.32 ± 0.36	9.74 ± 0.92	15.54 ± 0.45	8.39 ± 0.28	15.65 ± 0.62	10.03 ± 0.68	14.86 ± 0.91	8.92 ± 1.23
Kochi	33.57	9.69 ± 0.67	7.28 ± 0.76	10.23 ± 0.49	9.03 ± 0.34	11.48 ± 0.45	7.89 ± 0.40	11.78 ± 0.63	9.42 ± 0.75	10.77 ± 1.07	8.38 ± 1.10
Oita	33.24	12.46 ± 0.43	8.06 ± 0.62	12.63 ± 0.45	9.90 ± 0.53	13.76 ± 0.39	8.82 ± 0.32	14.12 ± 0.62	10.57 ± 0.82	13.24 ± 0.88	9.32 ± 1.20
Miyazaki	31.94	13.16 ± 0.48	9.23 ± 0.71	13.63 ± 0.18	11.18 ± 0.78	14.73 ± 0.54	9.81 ± 0.31	14.74 ± 0.65	11.29 ± 0.52	14.03 ± 0.87	10.35 ± 1.09
Kagoshima	31.56	15.46 ± 0.58	10.56 ± 0.80	15.82 ± 0.53	12.70 ± 0.95	17.20 ± 0.65	11.07 ± 0.62	17.36 ± 0.70	13.02 ± 0.63	16.43 ± 1.08	11.80 ± 1.34

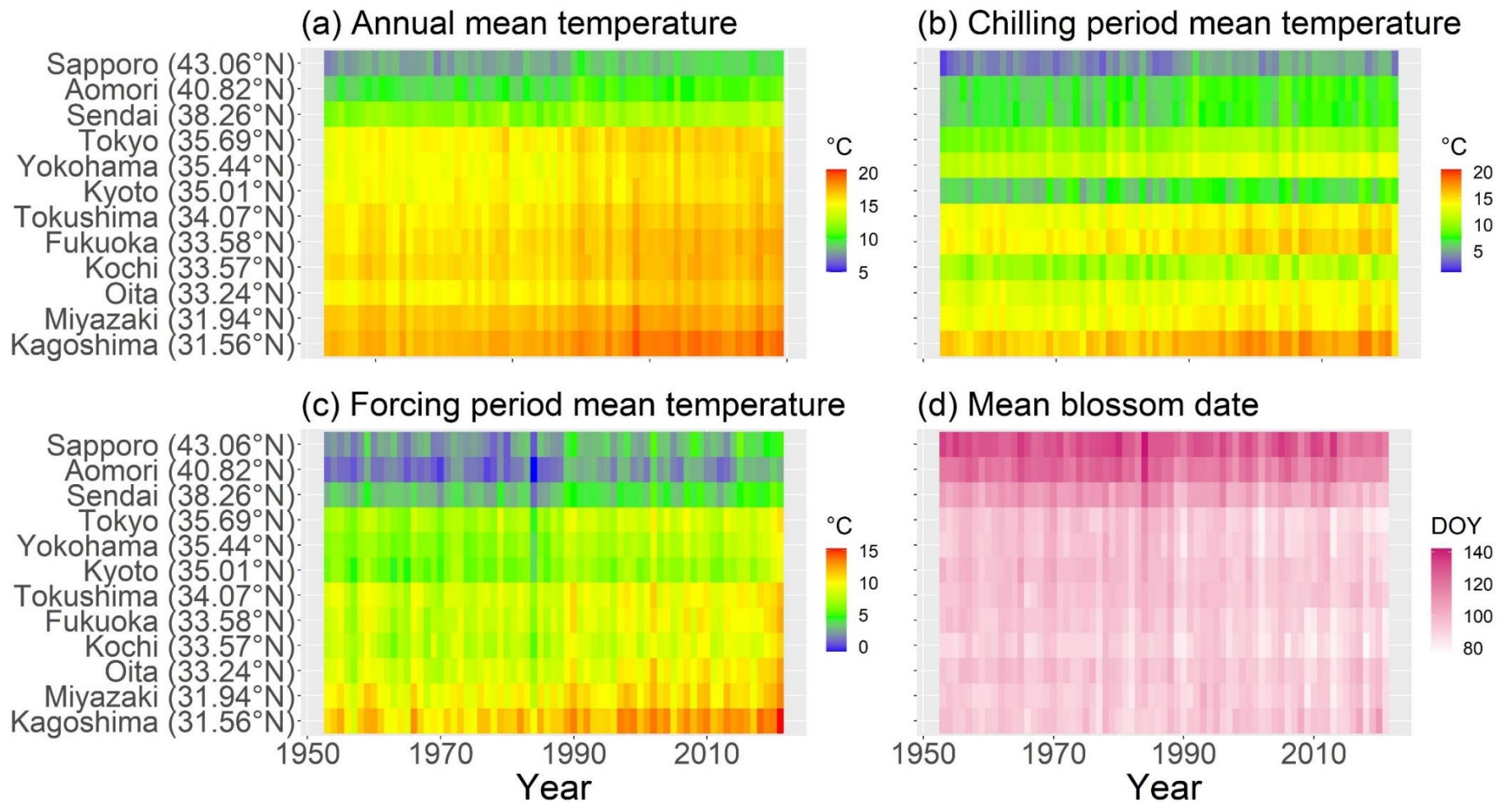


Figure SA2.1. Annual mean temperatures (a), chilling period mean temperatures (b), forcing period mean temperatures (c), and mean bloom dates (d) from high (Sapporo, 43.06°N) to low (Kagoshima, 31.56°N) latitudes between 1953 and 2021 in Japan. DOY: Day of the year.

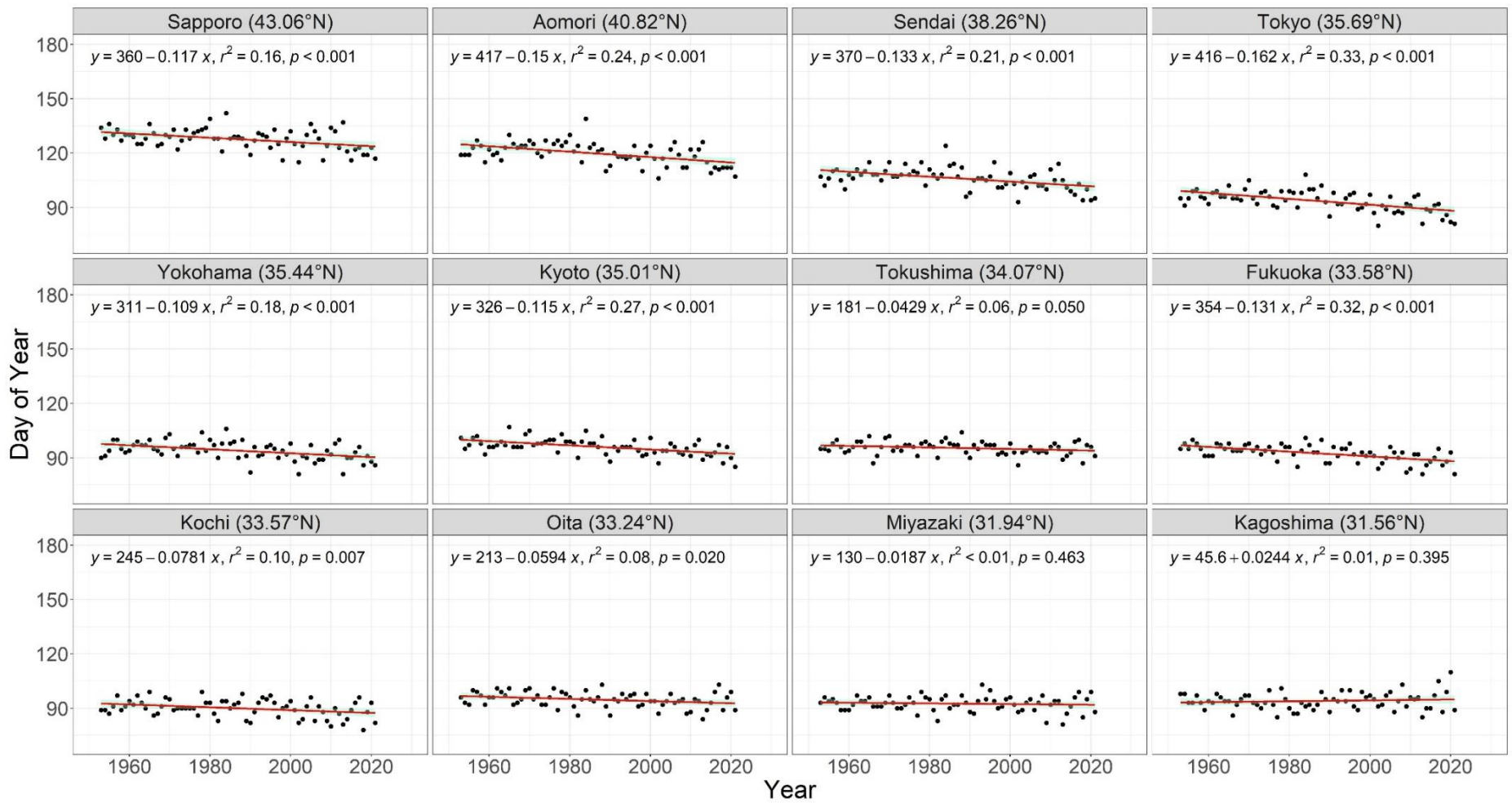


Figure SA2.2. Yoshino cherry bloom dates from 1953 to 2021 and regression lines across the latitudinal gradient from the highest (Sapporo, 43.06°N) to lowest (Kagoshima, 31.56°N) latitudes.

Table SA2.4. Average bloom dates and standard errors of Yoshino cherry of all years and deviation from the average bloom date of all years in different chilling-forcing temperature combinations (CC-CF: cold chilling-cold forcing; CC-WF: cold chilling-warm forcing; WC-CF: warm chilling-cold forcing; WC-WF: warm chilling-warm forcing) and ANOVA results over the entire study period from 1953 to 2021 in different locations and latitudes.

Location	Latitude (°N)	Average bloom date of all years	Deviation from the average bloom date of all years				Different combinations p-value
			CC-CF	CC-WF	WC-CF	WC-WF	
Sapporo	43.06	127.7 ± 0.7	3.6 ± 0.7 <sup>a</sup>	-3.0 ± 1.0 <sup>b</sup>	3.6 ± 1.4 <sup>a</sup>	-4.1 ± 1.1 <sup>b</sup>	≤0.001 <sup>***</sup>
Aomori	40.82	119.7 ± 0.7	4.2 ± 0.9 <sup>a</sup>	-7.1 ± 1.5 <sup>c</sup>	4.3 ± 0.9 <sup>a</sup>	-3.4 ± 0.9 <sup>b</sup>	≤0.001 <sup>***</sup>
Sendai	38.26	106.1 ± 0.7	3.7 ± 0.9 <sup>a</sup>	-5.2 ± 1.6 <sup>b</sup>	3.9 ± 1.0 <sup>a</sup>	-3.3 ± 0.9 <sup>b</sup>	≤0.001 <sup>***</sup>
Tokyo	35.69	93.6 ± 0.7	3.5 ± 0.9 <sup>a</sup>	-4.3 ± 1.6 <sup>b</sup>	4.5 ± 0.6 <sup>a</sup>	-3.7 ± 0.9 <sup>b</sup>	≤0.001 <sup>***</sup>
Yokohama	35.44	94.0 ± 0.6	2.5 ± 0.8 <sup>a</sup>	-4.1 ± 1.3 <sup>b</sup>	4.0 ± 1.3 <sup>a</sup>	-2.6 ± 0.9 <sup>b</sup>	≤0.001 <sup>***</sup>
Kyoto	35.01	96.1 ± 0.5	2.4 ± 0.8 <sup>a</sup>	-3.6 ± 1.2 <sup>b</sup>	2.9 ± 1.0 <sup>a</sup>	-2.2 ± 0.7 <sup>b</sup>	≤0.001 <sup>***</sup>
Tokushima	34.07	95.4 ± 0.4	1.8 ± 0.5 <sup>a</sup>	-4.8 ± 0.8 <sup>c</sup>	2.8 ± 0.6 <sup>a</sup>	-0.8 ± 0.6 <sup>b</sup>	≤0.001 <sup>***</sup>
Fukuoka	33.58	92.5 ± 0.6	2.8 ± 0.6 <sup>a</sup>	-3.2 ± 1.4 <sup>b</sup>	2.8 ± 0.7 <sup>a</sup>	-2.7 ± 0.9 <sup>b</sup>	≤0.001 <sup>***</sup>
Kochi	33.57	90.0 ± 0.6	1.4 ± 0.8 <sup>ab</sup>	-4.8 ± 1.0 <sup>c</sup>	3.7 ± 0.9 <sup>a</sup>	-0.6 ± 1.0 <sup>b</sup>	≤0.001 <sup>***</sup>
Oita	33.24	94.7 ± 0.5	2.2 ± 0.6 <sup>a</sup>	-3.9 ± 0.78 <sup>c</sup>	2.4 ± 0.8 <sup>a</sup>	-1.3 ± 1.0 <sup>b</sup>	≤0.001 <sup>***</sup>
Miyazaki	31.94	92.5 ± 0.5	0.4 ± 0.6 <sup>b</sup>	-3.5 ± 0.92 <sup>c</sup>	4.1 ± 0.7 <sup>a</sup>	-0.9 ± 0.9 <sup>b</sup>	≤0.001 <sup>***</sup>
Kagoshima	31.56	94.0 ± 0.6	-0.5 ± 0.6 <sup>b</sup>	-4.0 ± 0.97 <sup>c</sup>	4.5 ± 1.0 <sup>a</sup>	0.4 ± 1.1 <sup>b</sup>	≤0.001 <sup>***</sup>

Different letters (a, b, c) indicate significant differences between different chilling-forcing temperature combinations (row-wise comparisons) by

using Fisher's Protected Least Significant Difference (LSD). \*:  $p \leq 0.05$ ; \*\*:  $p \leq 0.001$ ; \*\*\*:  $p \leq 0.0001$

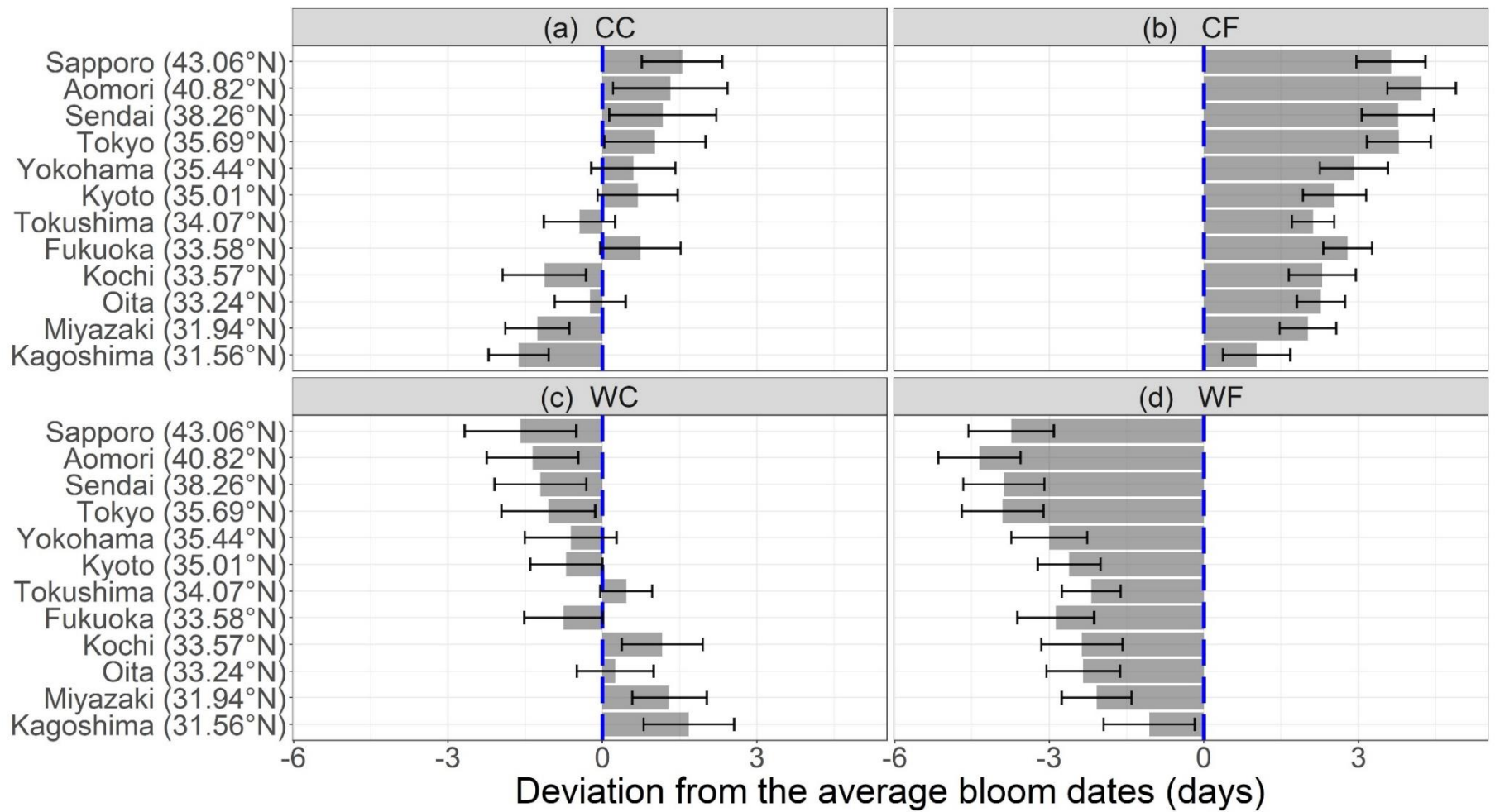


Figure SA2.3. The deviation of Yoshino cherry's bloom dates from the average bloom dates in different chilling and forcing temperature combinations over the entire study period from 1953 to 2021 in different latitudes and locations. (a) CC: cold chilling; (b) CF: cold forcing; (c) WC: warm chilling; (d) WF: warm forcing. Positive values mean the delay of the bloom date, and negative values indicate the advance of the bloom date. The error bars are standard errors.

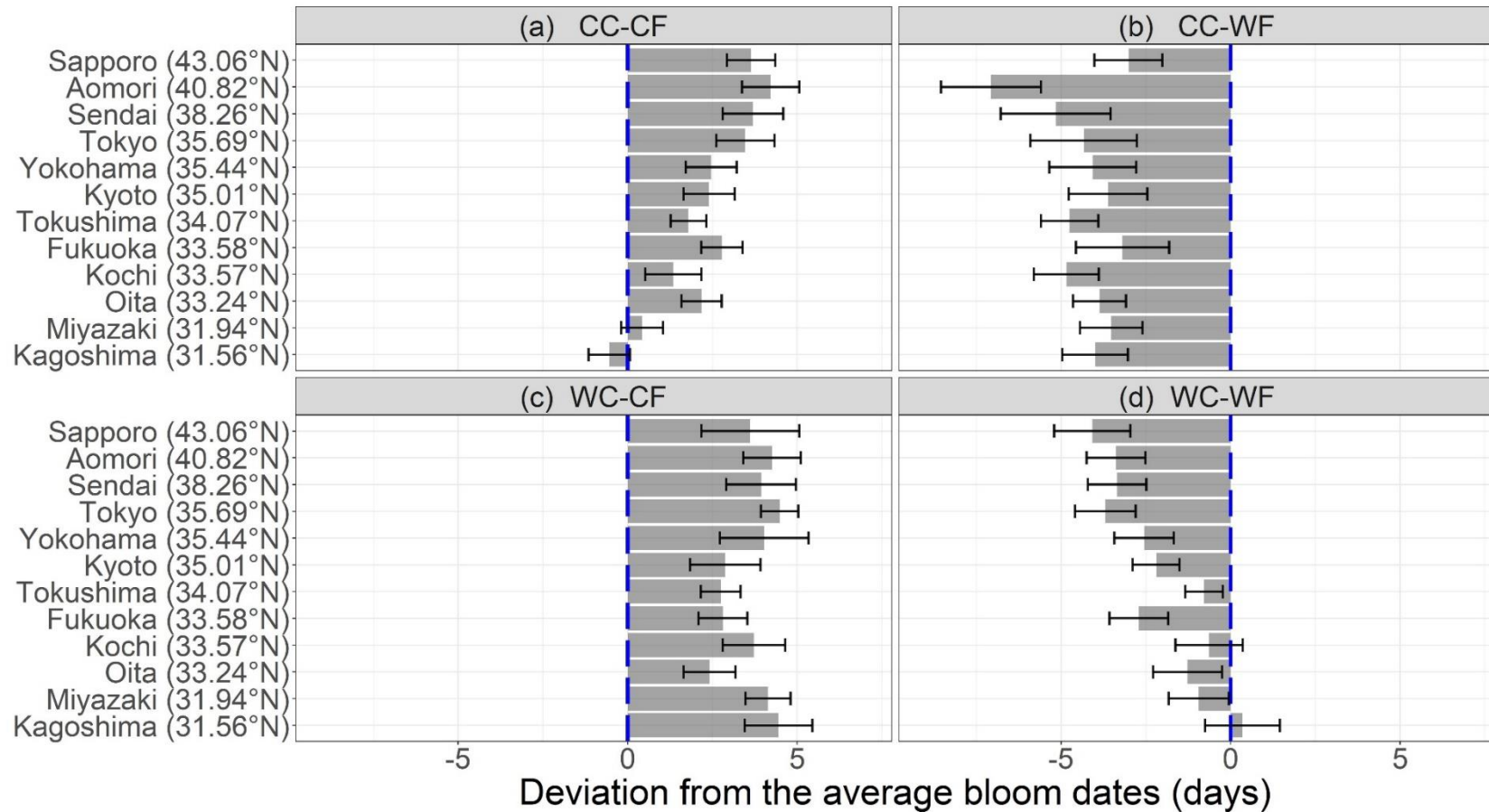


Figure SA2.4. The deviation of Yoshino cherry's bloom dates from the average bloom dates in four chilling-forcing combinations over the entire study period from 1953 to 2021. (a) CC-CF: cold chilling-cold forcing; (b) CC-WF: cold chilling-warm forcing; (c) WC-CF: warm chilling-cold forcing; (d) WC-WF: warm chilling-warm forcing in different latitudes and locations. Positive values mean the delay of the bloom date, and negative values indicate the advance of the bloom date. The error bars are standard errors.

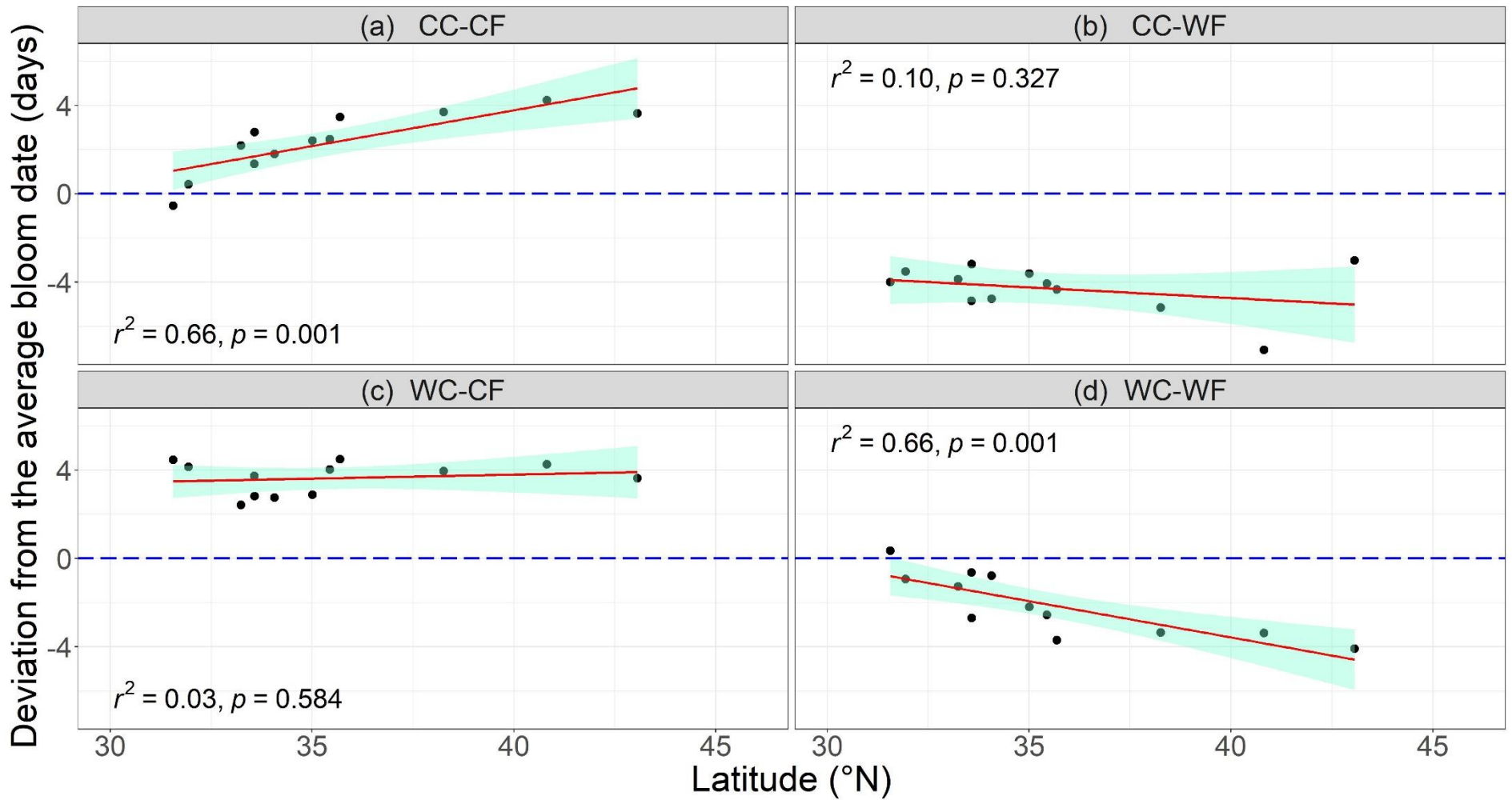


Figure SA2.5. A simple linear regression of the deviation of Yoshino cherry's bloom dates from the average bloom dates in four different chilling-forcing combinations across the latitudinal gradient over the entire study period from 1953 to 2021. (a) CC-CF: cold chilling-cold forcing; (b) CC-WF: cold chilling-warm forcing; (c) WC-CF: warm chilling-cold forcing; (d) WC-WF: warm chilling-warm forcing. Blue shadows are 95% confidence intervals.

Table SA2.5. Temperature sensitivity ( $S_T$ , day/°C) of chilling and forcing temperatures on Yoshino cherry's bloom dates over the entire study period from 1953 to 2021 along a latitudinal gradient. These effects were calculated as the ratio of  $\Delta$  bloom date and  $\Delta$  mean chilling or forcing temperature based on the comparisons described in the materials and methods.

		Temperature sensitivity ( $S_T$ ) of forcing temperature					
		after cold chilling temperature			after warm chilling temperature		
Locations	Latitudes (°N)	$\Delta$ Bloom date (day)	$\Delta$ Mean forcing temperature (°C)	Temperature sensitivity ( $S_T$ , day/°C)	$\Delta$ Bloom date (day)	$\Delta$ Mean forcing temperature (°C)	Temperature sensitivity ( $S_T$ , day/°C)
Sapporo	43.06	-6.66	1.47	-4.52	-7.71	1.75	-4.41
Aomori	40.82	-11.29	1.74	-6.48	-7.64	1.45	-5.28
Sendai	38.26	-8.86	1.46	-6.06	-7.29	1.31	-5.57
Tokyo	35.69	-7.81	1.74	-4.48	-8.18	1.50	-5.44
Yokohama	35.44	-6.54	1.54	-4.24	-6.58	1.43	-4.62
Kyoto	35.01	-6.02	1.55	-3.88	-5.08	1.35	-3.76
Tokushima	34.07	-6.55	1.96	-3.34	-3.53	1.39	-2.55
Fukuoka	33.58	-5.97	2.04	-2.92	-5.52	1.64	-3.36
Kochi	33.57	-6.19	1.75	-3.54	-4.36	1.53	-2.85
Oita	33.24	-6.05	1.84	-3.28	-3.69	1.75	-2.11
Miyazaki	31.94	-3.95	1.95	-2.03	-5.09	1.48	-3.44
Kagoshima	31.56	-3.46	2.14	-1.61	-4.11	1.94	-2.11

		Temperature sensitivity ( $S_T$ ) of chilling temperature					
		followed by cold forcing temperature			followed by warm forcing temperature		
Locations	Latitudes (°N)	$\Delta$ Bloom date (day)	$\Delta$ Mean chilling temperature (°C)	Temperature sensitivity ( $S_T$ , day/°C)	$\Delta$ Bloom date (day)	$\Delta$ Mean chilling temperature (°C)	Temperature sensitivity ( $S_T$ , day/°C)
Sapporo	43.06	-0.02	1.45	-0.01	-1.07	1.53	-0.70
Aomori	40.82	0.04	1.44	0.03	3.69	1.27	2.92

Sendai	38.26	0.24	1.52	0.16	1.81	1.53	1.18
Tokyo	35.69	1.01	1.62	0.62	0.64	1.40	0.46
Yokohama	35.44	1.56	1.44	1.08	1.52	1.35	1.12
Kyoto	35.01	0.48	1.47	0.33	1.42	1.37	1.04
Tokushima	34.07	0.95	1.34	0.71	3.97	1.38	2.87
Fukuoka	33.58	0.03	1.51	0.02	0.48	1.34	0.36
Kochi	33.57	2.38	1.80	1.33	4.21	1.55	2.72
Oita	33.24	0.24	1.29	0.18	2.59	1.48	1.75
Miyazaki	31.94	3.72	1.57	2.37	2.58	1.11	2.33
Kagoshima	31.56	5.00	1.74	2.88	4.35	1.54	2.82

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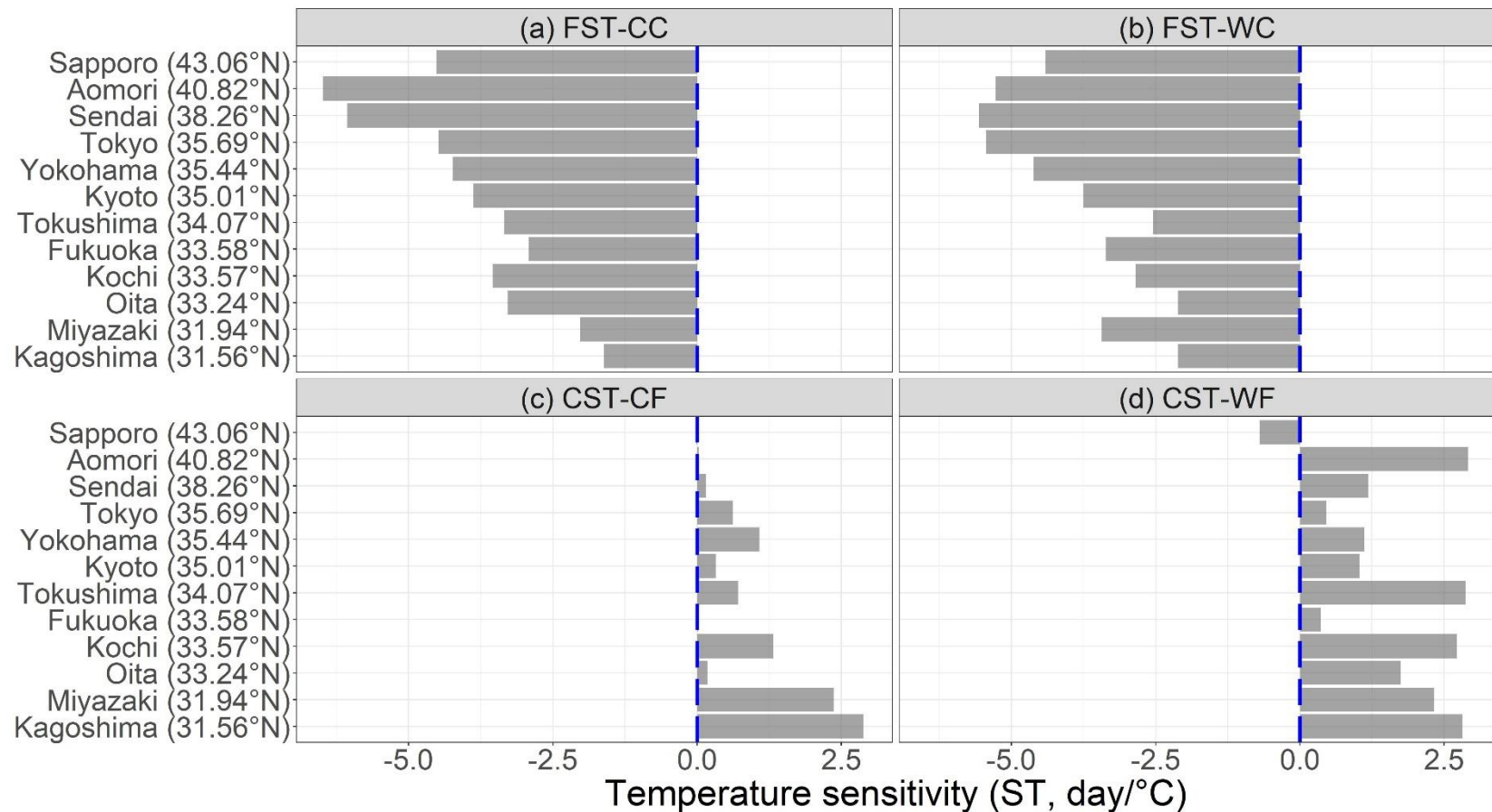


Figure SA2.6. Temperature sensitivity ( $S_T$ , day/°C) of forcing and chilling temperatures on Yoshino cherry bloom dates over the entire study period from 1953 to 2021 along a latitudinal gradient.  $S_T$  was calculated as the ratio of  $\Delta$  bloom date and  $\Delta$  mean forcing or chilling temperature based on the comparisons described in the materials and methods. (a) FST-CC:  $S_T$  in forcing period after cold chilling temperature; (b) FST-WC:  $S_T$  in forcing period after warm chilling temperature; (c) CST-CF:  $S_T$  in chilling period followed by cold forcing temperature; (d) CST-WF:  $S_T$  in chilling period followed by warm forcing temperature.

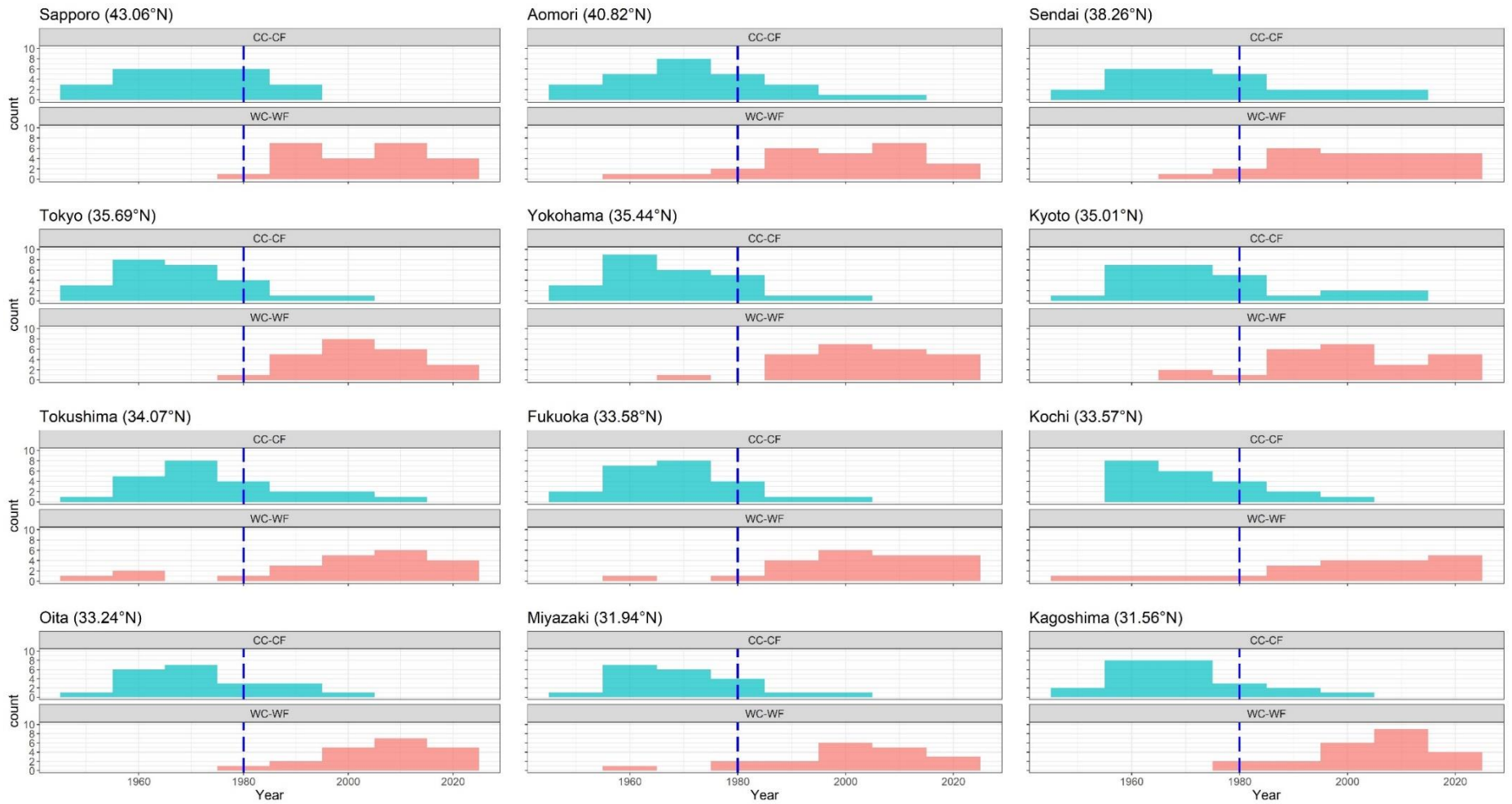


Figure SA2.7. The frequency of years that were classified as cold chilling-cold forcing (CC-CF) and warm chilling-warm forcing (WC-WF) from the highest (Sapporo, 43.06°N) to lowest (Kagoshima, 31.56°N) latitudes. The blue dash line is the year of 1980.

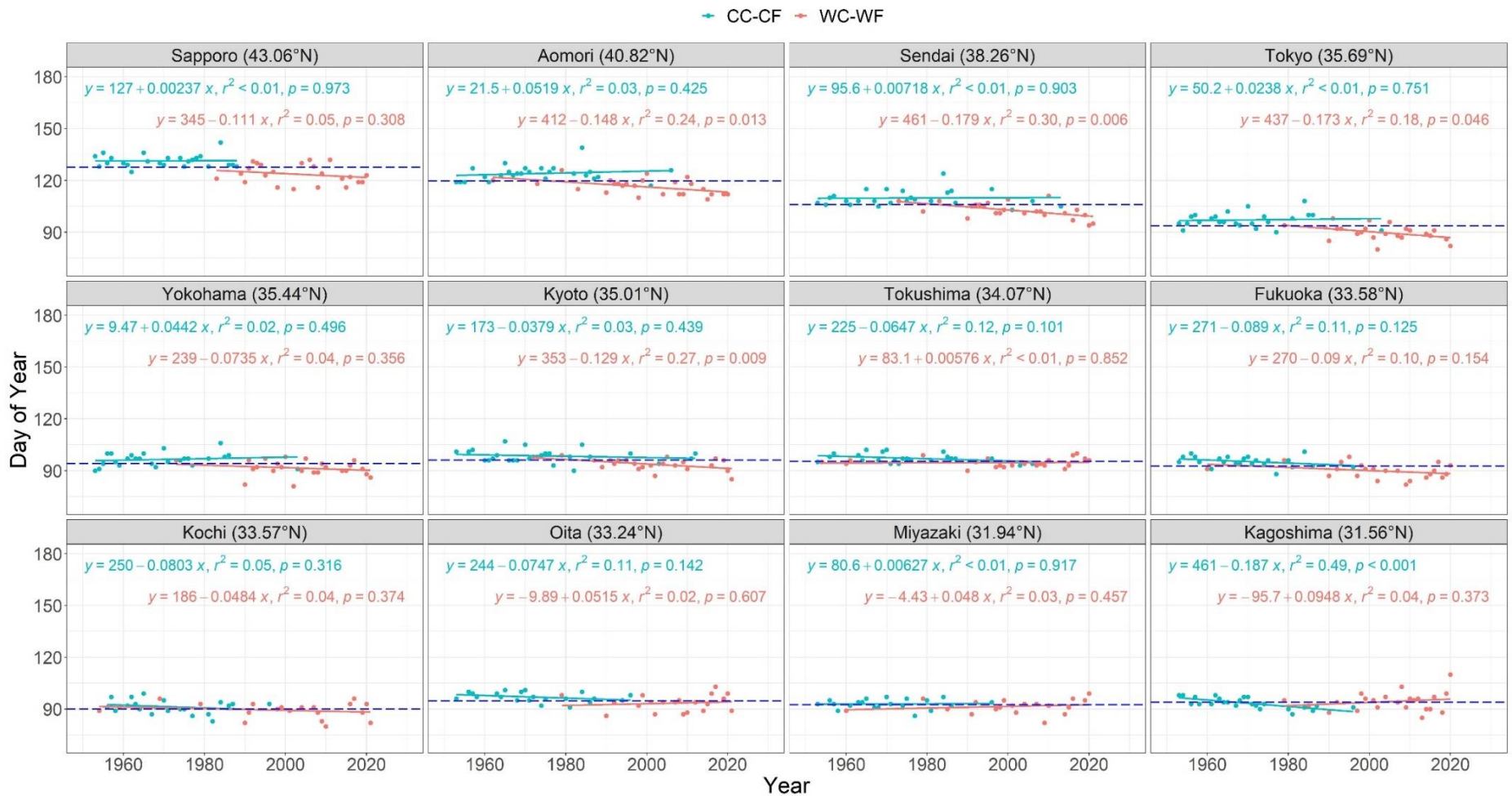


Figure SA2.8. Yoshino cherry bloom dates of cold chilling-cold forcing (CC-CF) and warm chilling-warm forcing (WC-WF) and regression lines across the latitudinal gradient from the highest (Sapporo, 43.06°N) to lowest (Kagoshima, 31.56°N) latitudes. The dashed blue lines are the average bloom dates of each location from 1953 to 2021.

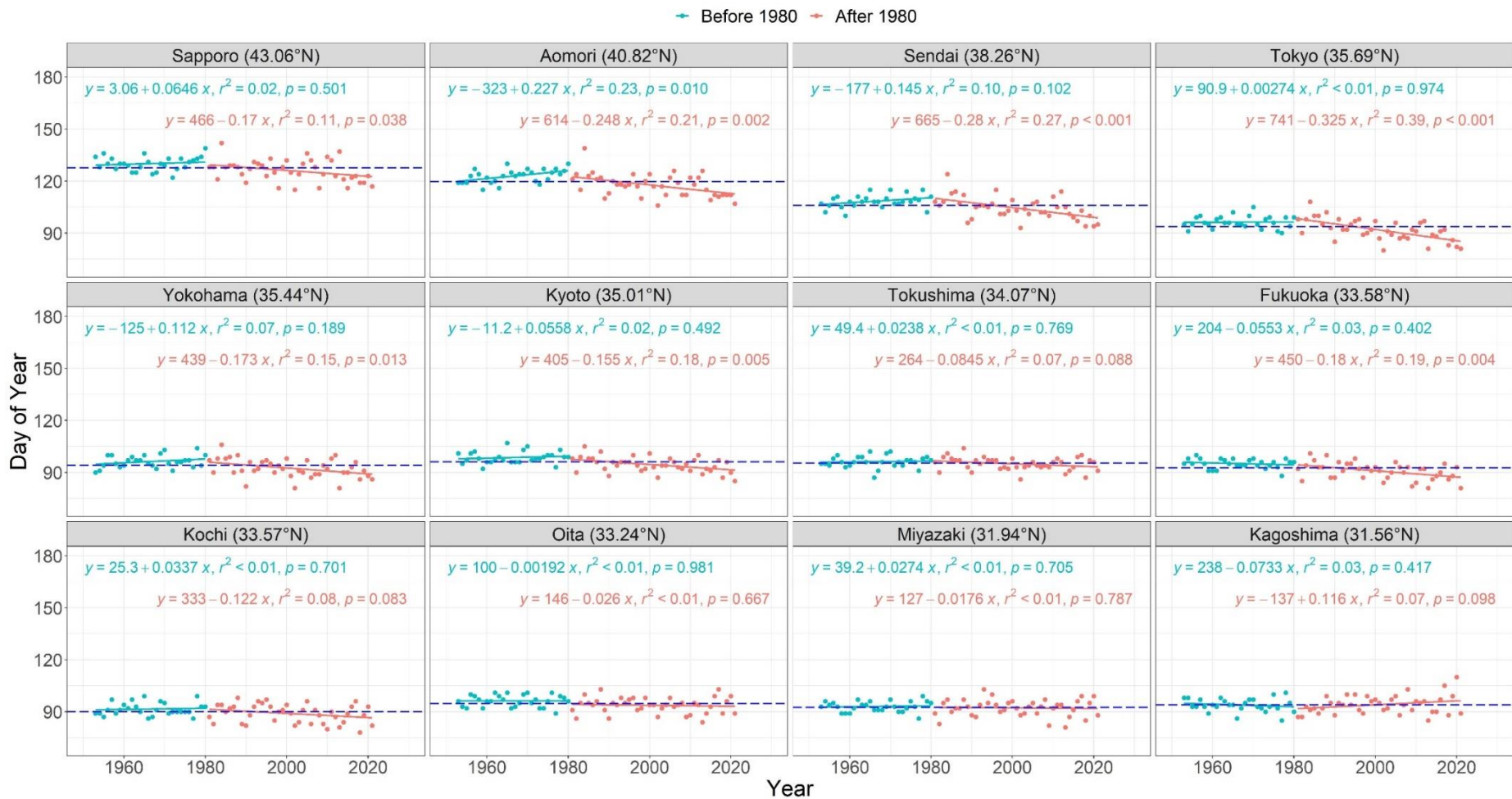


Figure SA2.9. Yoshino cherry bloom dates before and after 1980 and regression lines across the latitudinal gradient from the highest (Sapporo, 43.06°N) to lowest (Kagoshima, 31.56°N) latitudes. The dashed blue lines are the average bloom dates of each location from 1953 to 2021.

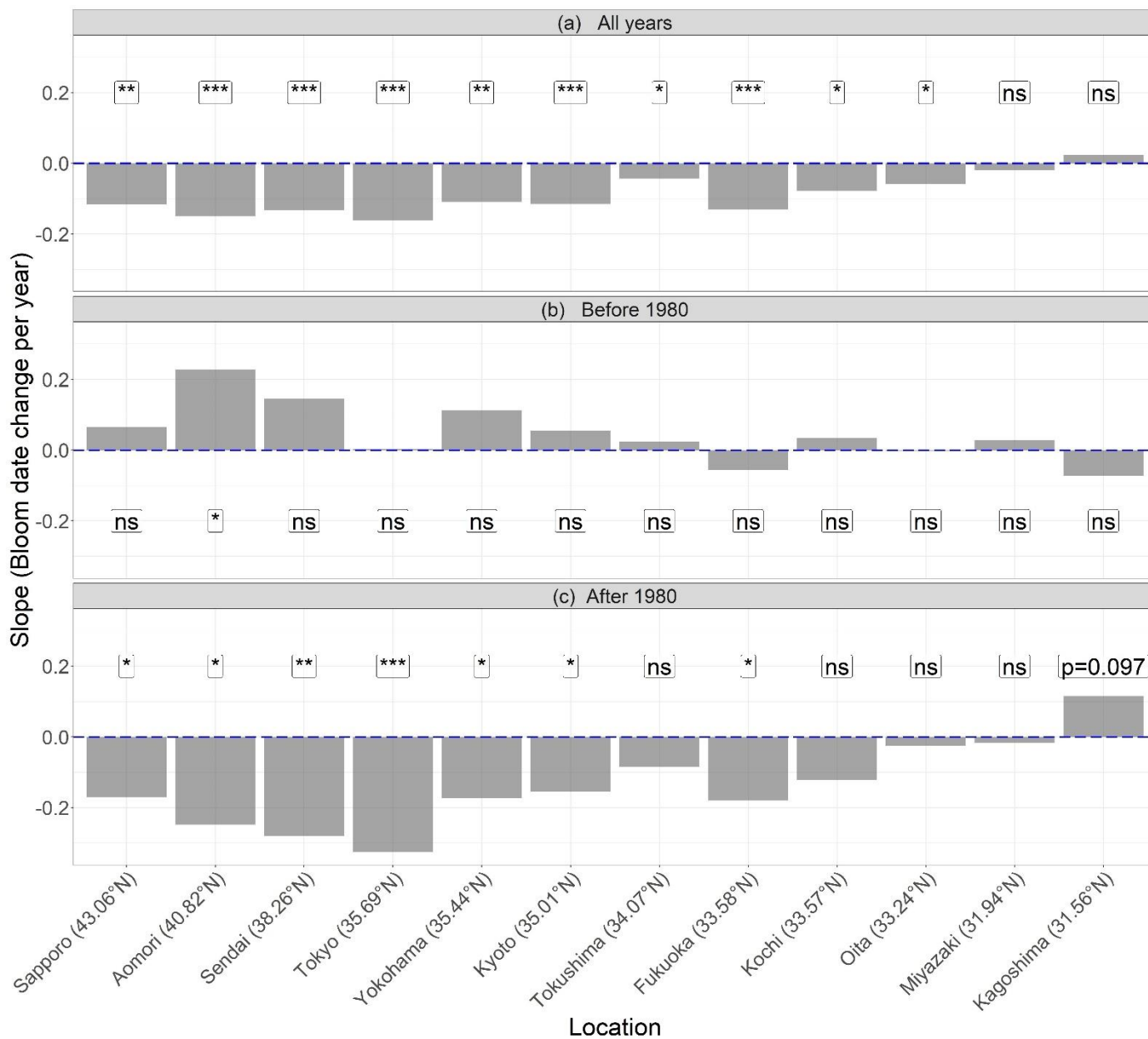


Figure SA10. The bloom date change per year of (a) all years (slopes in Figure SA2); (b) before 1980 (slope before 1980 in Figure SA8); (c) after 1980 (slopes after 1980 in Figure SA8) across the latitudinal gradient from the highest (Sapporo, 43.06°N) to lowest (Kagoshima, 31.56°N) latitudes. Positive slope: delay of the bloom date; Negative slope: advance of the bloom date. ns: non-significant; \*:  $p \leq 0.05$ ; \*\*:  $p \leq 0.01$ ; \*\*\*:  $p \leq 0.001$

## Supplementary materials B

### 1. Calculation of chilling and forcing requirements

The Dynamic Model hypothesizes that winter chill accumulates in a two-step process. At first, cold temperatures result in the formation of an intermediate product. However, it could be destroyed by high temperatures. Once a certain amount of this intermediate product has accumulated, it can be converted into a Chill Portion by a process requiring relatively moderate temperatures. The equations used to calculate Chill Portions are more complex than the other models (Luedeling *et al.*, 2009a).

$$x_i = \frac{e^{(slp \cdot tetmlt)((T_K - tetmlt)/T_K)}}{1 + e^{(slp \cdot tetmlt)((T_K - tetmlt)/T_K)}}$$

$$x_s = \frac{a_0}{a_1} \cdot e^{(e_1 - e_0)/T_K}$$

$$ak_1 = a_1 \cdot e^{-(e_1 - e_0)/T_K}$$

$$inter_E = x_s - (x_s - inter_s) \cdot e^{-ak_1}$$

$$inter_s = \begin{cases} t = t_0 & : 0 \\ t > t_0 \wedge inter_{E_{T-1}} < 1 & : inter_{E_{t-1}} \\ t > t_0 \wedge inter_{E_{T-1}} \geq 1 & : inter_{E_{t-1}} \cdot (1 - x_i) \end{cases}$$

$$delt = \begin{cases} t = t_0 & : 0 \\ t > t_0 \wedge inter_E < 1 & : 0 \\ t > t_0 \wedge inter_E \geq 1 & : x_i \cdot inter_E \end{cases}$$

$$Chill\ Portions_t = \begin{cases} t = t_0 & : delt \\ t \geq t_0 & : delt + Chill\ Portions_{t-1} \end{cases}$$

The constants **slp**, **tetmlt**, **a<sub>0</sub>**, **a<sub>1</sub>**, **e<sub>0</sub>** and **e<sub>1</sub>** were set to 1.6, 277, 139500,  $2.567 \times 10^{18}$ , 12888.8 and 4153.5, respectively, according to Luedeling *et al.* (2009a). **T<sub>k</sub>** is the measured hourly temperature in Kelvin, while **t** denotes the time during the season (in hours), with **t<sub>0</sub>** being the starting point of chilling accumulation. Growing Degree Hours (GDH; Anderson *et al.*, 1986) were calculated from hourly temperatures (**T<sub>h</sub>**), as a function of a base (**T<sub>b</sub>**), an optimum (**T<sub>u</sub>**) and a critical (**T<sub>c</sub>**) temperature. The function is given in Luedeling *et al.* (2009b). The underlying assumption is that heat accumulates, when temperatures range between **T<sub>b</sub>** and **T<sub>c</sub>**, with maximum accumulation at **T<sub>u</sub>**. For temperatures between **T<sub>b</sub>** and **T<sub>u</sub>**, the corresponding equation is:

$$GDH = F \frac{T_u - T_b}{2} \left( 1 + \cos \left( \pi + \pi \frac{T_h - T_b}{T_u - T_b} \right) \right)$$

whereas for temperatures between **T<sub>u</sub>** and **T<sub>c</sub>**, heat accumulates as:

$$GDH = F(T_u - T_b) \left( 1 + \cos \left( \frac{\pi}{2} + \frac{\pi T_h - T_u}{2 T_c - T_u} \right) \right)$$

In both equations, **F** is a plant stress factor that is commonly set to 1, if no particular stresses are assumed. **T<sub>b</sub>**, **T<sub>u</sub>** and **T<sub>c</sub>** were set to 4, 25 and 36 °C, respectively, as suggested by Anderson *et al.* (1986) for fruit trees.

## 2. Chilling and forcing requirements for Yoshino cherry to bloom across the latitudinal gradient

The chilling requirements decreased with decreasing latitudes for all years and combinations (Table SB2.1 and SB2.2). As expected, cold or warm forcing temperatures did not significantly affect the chilling requirements at all locations (Table SB2.2). We classified cold or warm chilling periods based on above or below the average temperature in these periods; supposedly, the chilling requirements should be significantly different between cold and warm chilling combinations, but interestingly, it was not the case at the higher latitudes (Sapporo, Aomori, Sendai, and Kyoto; Table SB2.2). The ANOVA revealed the same trend as the two-way ANOVA that cold

or warm chilling and forcing temperatures did not have significant effects on the chilling requirements at higher latitude locations (Sapporo, Aomori, Sendai, and Kyoto; Table SB2.1), and the chilling requirements were similar in the combinations that had cold chilling temperatures (CC-CF, CC-WF) and also in the combinations that had warm chilling temperatures (WC-CF, WC-WF), irrespective of the forcing temperatures (Table SB2.1). The forcing requirements were inversely related to latitude for all years and combinations (Tables SB2.3 and SB2.4). As expected, cold or warm forcing temperatures significantly affected the forcing requirements at all locations ( $p \leq 0.001$ ; Table SB2.4). Unlike the forcing temperatures imposing little effect on chilling requirements (Table SB2.2), the warm chilling temperatures led to greater forcing requirements at most latitudes ( $p \leq 0.05$ ,  $p \leq 0.01$ , or  $p \leq 0.001$ ), except for Sapporo (Table SB2.4). Both chilling and forcing temperatures affected forcing requirements, resulting in forcing requirements being significantly different in four chilling-forcing temperature combinations (CC-CF, CC-WF, WC-CF, WC-WF) at all latitudes ( $p \leq 0.001$ ; Table SB2.3).

Table SB2.1. Deviation from the average chilling requirement of all years (unit: Chill Portion, CP) of Yoshino cherry in all years and different chilling-forcing temperature combinations (CC-CF: cold chilling-cold forcing, CC-WF: cold chilling-warm forcing, WC-CF: warm chilling-cold forcing, WC-WF: warm chilling-warm forcing), and ANOVA results over the entire study period from 1953 to 2021 along latitudes. Values behind  $\pm$  are standard errors. CV: Coefficient of Variance (in parentheses).

Location	Latitude (°N)	Average chilling requirement of all years	Deviation from the average chilling requirement of all years (Chill Portion)				
			CC-CF	CC-WF	WC-CF	WC-WF	Different combinations p-value
Sapporo	43.06	59.9 $\pm$ 0.7 (9.4)	-1.4 $\pm$ 1.2 (10.2)	1.5 $\pm$ 1.4 (7.6)	0.1 $\pm$ 1.5 (8.0)	0.8 $\pm$ 1.2 (9.8)	0.433
Aomori	40.82	52.0 $\pm$ 0.5 (8.3)	-0.3 $\pm$ 0.9 (8.5)	0.8 $\pm$ 1.9 (10.9)	-0.9 $\pm$ 1.2 (6.9)	0.3 $\pm$ 0.8 (7.8)	0.824
Sendai	38.26	47.3 $\pm$ 0.4 (6.5)	0.0 $\pm$ 0.6 (6.5)	2.0 $\pm$ 0.9 (5.5)	-1.1 $\pm$ 0.7 (4.8)	-0.3 $\pm$ 0.7 (7.1)	0.133
Tokyo	35.69	56.1 $\pm$ 0.5 (7.0)	2.6 $\pm$ 0.6 <sup>a</sup> (5.1)	2.0 $\pm$ 1.0 <sup>a</sup> (5.9)	-3.0 $\pm$ 1.0 <sup>b</sup> (6.0)	-2.3 $\pm$ 0.6 <sup>b</sup> (5.5)	$\leq$ 0.001 <sup>***</sup>
Yokohama	35.44	39.5 $\pm$ 0.5 (10.6)	3.3 $\pm$ 0.5 <sup>a</sup> (6.0)	2.3 $\pm$ 1.0 <sup>a</sup> (7.7)	-2.7 $\pm$ 1.1 <sup>b</sup> (9.2)	-3.1 $\pm$ 0.6 <sup>b</sup> (8.5)	$\leq$ 0.001 <sup>***</sup>
Kyoto	35.01	57.5 $\pm$ 0.3 (4.8)	0.3 $\pm$ 0.6 (5.0)	1.0 $\pm$ 0.9 (5.1)	-0.5 $\pm$ 0.7 (3.7)	-0.5 $\pm$ 0.6 (4.8)	0.449
Tokushima	34.07	38.1 $\pm$ 0.5 (11.2)	2.9 $\pm$ 0.7 <sup>a</sup> (8.6)	2.5 $\pm$ 1.1 <sup>a</sup> (9.6)	-3.3 $\pm$ 0.7 <sup>b</sup> (7.3)	-2.7 $\pm$ 0.6 <sup>b</sup> (8.2)	$\leq$ 0.001 <sup>***</sup>
Fukuoka	33.58	37.2 $\pm$ 0.5 (11.2)	3.5 $\pm$ 0.4 <sup>a</sup> (5.1)	1.9 $\pm$ 1.1 <sup>a</sup> (9.9)	-3.0 $\pm$ 0.7 <sup>b</sup> (6.7)	-2.9 $\pm$ 0.7 <sup>b</sup> (10.1)	$\leq$ 0.001 <sup>***</sup>
Kochi	33.57	34.1 $\pm$ 0.5 (12.7)	3.5 $\pm$ 0.8 <sup>a</sup> (9.2)	2.2 $\pm$ 1.1 <sup>a</sup> (10.9)	-3.0 $\pm$ 0.6 <sup>b</sup> (7.2)	-3.0 $\pm$ 0.6 <sup>b</sup> (8.7)	$\leq$ 0.001 <sup>***</sup>
Oita	33.24	37.7 $\pm$ 0.5 (11.0)	2.9 $\pm$ 0.6 <sup>a</sup> (6.4)	2.7 $\pm$ 1.1 <sup>a</sup> (10.6)	-2.5 $\pm$ 0.7 <sup>b</sup> (7.4)	-3.0 $\pm$ 0.7 <sup>b</sup> (8.5)	$\leq$ 0.001 <sup>***</sup>
Miyazaki	31.94	35.3 $\pm$ 0.5 (12.7)	3.1 $\pm$ 0.9 <sup>a</sup> (10.0)	1.8 $\pm$ 1.0 <sup>a</sup> (10.5)	-3.3 $\pm$ 0.7 <sup>b</sup> (8.4)	-2.1 $\pm$ 0.9 <sup>b</sup> (11.6)	$<$ 0.001 <sup>***</sup>
Kagoshima	31.56	33.1 $\pm$ 0.6 (15.6)	3.3 $\pm$ 0.7 <sup>a</sup> (10.0)	1.6 $\pm$ 1.4 <sup>a</sup> (13.2)	-3.1 $\pm$ 1.2 <sup>b</sup> (13.1)	-2.7 $\pm$ 1.1 <sup>b</sup> (17.1)	$<$ 0.001 <sup>***</sup>

Different letters (a, b, c) indicate significant differences between different chilling-forcing temperature combinations (row-wise comparisons) by using Fisher's Protected Least Significant Difference (LSD). \*:  $p \leq 0.05$ ; \*\*:  $p \leq 0.01$ ; \*\*\*:  $p \leq 0.001$

Table SB2.2. Deviation from the average chilling requirement of all years (unit: Chill Portion, CP) of Yoshino cherry in all years and in different chilling and forcing temperatures (CC: cold chilling, WC: warm chilling, CF: cold forcing, WF: warm forcing), and two-way ANOVA results of chilling and forcing temperatures over the entire study period from 1953 to 2021 in different latitudes and latitudes. Values behind  $\pm$  are standard errors. CV: Coefficient of Variance (in parentheses).

Location	Latitude (°N)	Average chilling requirement of all years	Deviation from the average chilling requirement of all years (Chill Portion)					
			CC	WC	Chilling temperature p-value	CF	WF	Forcing temperature p-value
Sapporo	43.06	59.9 $\pm$ 0.7 (9.4)	-0.5 $\pm$ 1.0 (9.6)	0.6 $\pm$ 1.0 (9.2)	0.427	-0.9 $\pm$ 0.9 (9.5)	1.0 $\pm$ 0.9 (9.1)	0.218
Aomori	40.82	52.0 $\pm$ 0.5 (8.3)	0.0 $\pm$ 0.8 (9.1)	0.0 $\pm$ 0.7 (7.5)	0.965	-0.4 $\pm$ 0.7 (8.0)	0.5 $\pm$ 0.8 (8.5)	0.343
Sendai	38.26	47.3 $\pm$ 0.4 (6.5)	0.6 $\pm$ 0.5 (6.5)	-0.5 $\pm$ 0.5 (6.5)	0.141	-0.3 $\pm$ 0.5 (6.1)	0.4 $\pm$ 0.6 (6.9)	0.087
Tokyo	35.69	56.1 $\pm$ 0.5 (7.0)	2.4 $\pm$ 0.5 (5.3)	-2.5 $\pm$ 0.5 (5.6)	$\leq 0.001^{***}$	0.8 $\pm$ 0.7 (7.0)	-0.9 $\pm$ 0.6 (6.7)	0.930
Yokohama	35.44	39.5 $\pm$ 0.5 (10.6)	3.0 $\pm$ 0.5 (6.5)	-3.0 $\pm$ 0.5 (8.6)	$\leq 0.001^{***}$	1.6 $\pm$ 0.7 (9.4)	-1.5 $\pm$ 0.7 (10.5)	0.364
Kyoto	35.01	57.5 $\pm$ 0.3 (4.8)	0.5 $\pm$ 0.5 (5.0)	-0.5 $\pm$ 0.4 (4.4)	0.139	0.1 $\pm$ 0.5 (4.6)	-0.1 $\pm$ 0.5 (5.0)	0.653
Tokushima	34.07	38.1 $\pm$ 0.5 (11.2)	2.8 $\pm$ 0.6 (8.8)	-2.9 $\pm$ 0.5 (7.8)	$\leq 0.001^{***}$	0.8 $\pm$ 0.7 (11.2)	-0.9 $\pm$ 0.7 (11.0)	0.935
Fukuoka	33.58	37.2 $\pm$ 0.5 (11.2)	2.9 $\pm$ 0.5 (7.2)	-2.9 $\pm$ 0.5 (8.9)	$\leq 0.001^{***}$	1.3 $\pm$ 0.6 (9.8)	-1.2 $\pm$ 0.7 (11.8)	0.308
Kochi	33.57	34.1 $\pm$ 0.5 (12.7)	3.0 $\pm$ 0.6 (9.9)	-3.0 $\pm$ 0.4 (8.0)	$\leq 0.001^{***}$	0.9 $\pm$ 0.7 (12.6)	-0.9 $\pm$ 0.7 (12.4)	0.381
Oita	33.24	37.7 $\pm$ 0.5 (11.0)	2.8 $\pm$ 0.6 (8.2)	-2.8 $\pm$ 0.5 (8.0)	$\leq 0.001^{***}$	0.7 $\pm$ 0.6 (9.6)	-0.7 $\pm$ 0.8 (12.1)	0.620
Miyazaki	31.94	35.3 $\pm$ 0.5 (12.7)	2.5 $\pm$ 0.7 (10.2)	-2.7 $\pm$ 0.6 (10.4)	$\leq 0.001^{***}$	0.4 $\pm$ 0.8 (13.1)	-0.4 $\pm$ 0.7 (12.3)	0.921
Kagoshima	31.56	33.1 $\pm$ 0.6 (15.6)	2.7 $\pm$ 0.7 (11.1)	-2.8 $\pm$ 0.8 (15.7)	$\leq 0.001^{***}$	1.3 $\pm$ 0.8 (13.8)	-1.3 $\pm$ 0.9 (16.8)	0.552

\*:  $p \leq 0.05$ ; \*\*:  $p \leq 0.01$ ; \*\*\*:  $p \leq 0.001$

Table SB2.3. Deviation from the average forcing requirement of all years (unit: Growing Degree days, GDD) of Yoshino cherry in all years and different chilling-forcing temperature combinations (CC-CF: cold chilling-cold forcing, CC-WF: cold chilling-warm forcing, WC-CF: warm chilling-cold forcing, WC-WF: warm chilling-warm forcing), and ANOVA results over the entire study period from 1953 to 2021 along latitudes. Values behind  $\pm$  are standard errors. CV: Coefficient of Variance (in parentheses).

Location	Latitude (°N)	Average forcing requirement of all years	Deviation from the average forcing requirement of all years (GDD)				Different combinations p-value
			CC-CF	CC-WF	WC-CF	WC-WF	
Sapporo	43.06	3378 $\pm$ 128 (31.4)	-515 $\pm$ 152 <sup>b</sup> (26.7)	551 $\pm$ 134 <sup>a</sup> (11.9)	-702 $\pm$ 281 <sup>b</sup> (36.6)	610 $\pm$ 229 <sup>a</sup> (28.2)	<0.001 <sup>***</sup>
Aomori	40.82	3107 $\pm$ 110 (29.3)	-590 $\pm$ 107 <sup>c</sup> (22)	1056 $\pm$ 210 <sup>a</sup> (16.1)	-625 $\pm$ 89 <sup>c</sup> (11.5)	458 $\pm$ 163 <sup>b</sup> (23.4)	<0.001 <sup>***</sup>
Sendai	38.26	3501 $\pm$ 118 (28.1)	-590 $\pm$ 164 <sup>b</sup> (28.8)	731 $\pm$ 264 <sup>a</sup> (20.8)	-427 $\pm$ 169 <sup>b</sup> (18.3)	488 $\pm$ 174 <sup>a</sup> (21.9)	<0.001 <sup>***</sup>
Tokyo	35.69	4986 $\pm$ 173 (28.8)	-963 $\pm$ 194 <sup>b</sup> (24.2)	988 $\pm$ 474 <sup>a</sup> (27.6)	-1026 $\pm$ 122 <sup>b</sup> (10.7)	1023 $\pm$ 200 <sup>a</sup> (16.3)	<0.001 <sup>***</sup>
Yokohama	35.44	5610 $\pm$ 197 (29.2)	-1144 $\pm$ 200 <sup>b</sup> (22.8)	866 $\pm$ 320 <sup>a</sup> (16.5)	-1024 $\pm$ 168 <sup>b</sup> (12.2)	1257 $\pm$ 310 <sup>a</sup> (22.6)	<0.001 <sup>***</sup>
Kyoto	35.01	5419 $\pm$ 162 (24.8)	-973 $\pm$ 165 <sup>b</sup> (18.9)	962 $\pm$ 313 <sup>a</sup> (16.4)	-778 $\pm$ 188 <sup>b</sup> (13.5)	937 $\pm$ 237 <sup>a</sup> (18.7)	<0.001 <sup>***</sup>
Tokushima	34.07	4852 $\pm$ 132 (22.6)	-1009 $\pm$ 128 <sup>b</sup> (16.3)	1010 $\pm$ 266 <sup>a</sup> (16.4)	-608 $\pm$ 118 <sup>b</sup> (10.0)	836 $\pm$ 110 <sup>a</sup> (9.2)	<0.001 <sup>***</sup>
Fukuoka	33.58	6012 $\pm$ 198 (27.3)	-1428 $\pm$ 184 <sup>b</sup> (19.7)	1077 $\pm$ 435 <sup>a</sup> (22.2)	-935 $\pm$ 116 <sup>b</sup> (8.3)	1415 $\pm$ 207 <sup>a</sup> (13.4)	<0.001 <sup>***</sup>
Kochi	33.57	7264 $\pm$ 177 (20.2)	-1207 $\pm$ 216 <sup>b</sup> (16.7)	817 $\pm$ 138 <sup>a</sup> (6.6)	-895 $\pm$ 191 <sup>b</sup> (11.7)	1322 $\pm$ 268 <sup>a</sup> (14.3)	<0.001 <sup>***</sup>
Oita	33.24	4756 $\pm$ 140 (24.5)	-1086 $\pm$ 124 <sup>d</sup> (15.8)	531 $\pm$ 145 <sup>b</sup> (10.6)	-581 $\pm$ 117 <sup>c</sup> (10.9)	1175 $\pm$ 212 <sup>a</sup> (16.4)	<0.001 <sup>***</sup>
Miyazaki	31.94	8432 $\pm$ 182 (17.9)	-1325 $\pm$ 220 <sup>b</sup> (14.2)	1093 $\pm$ 326 <sup>a</sup> (13.7)	-955 $\pm$ 126 <sup>b</sup> (6.7)	1287 $\pm$ 170 <sup>a</sup> (7.8)	<0.001 <sup>***</sup>
Kagoshima	31.56	8024 $\pm$ 201 (20.8)	-1443 $\pm$ 192 <sup>b</sup> (14.6)	1091 $\pm$ 396 <sup>a</sup> (15.1)	-1049 $\pm$ 224 <sup>b</sup> (11.2)	1485 $\pm$ 179 <sup>a</sup> (9.2)	<0.001 <sup>***</sup>

Different letters (a, b, c) indicate significant differences between different chilling-forcing temperature combinations (row-wise comparisons) by using Fisher's Protected Least Significant Difference (LSD). \*:  $p \leq 0.05$ ; \*\*:  $p \leq 0.01$ ; \*\*\*:  $p \leq 0.001$

Table SB2.4. Deviation from the average forcing requirement of all years (unit: Growing Degree Days, GDD) of Yoshino cherry in all years and in different chilling and forcing temperatures (CC: cold chilling, WC: warm chilling, CF: cold forcing, WF: warm forcing), and two-way ANOVA results of chilling and forcing temperatures over the entire study period from 1953 to 2021 in different latitudes and locations. Values behind  $\pm$  are standard errors. CV: Coefficient of Variance (in parentheses).

Location	Latitude (°N)	Average forcing requirement of all years	Deviation from the average forcing requirement of all years (GDD)					
			CC	WC	Chilling temperature p-value	CF	WF	Forcing temperature p-value
Sapporo	43.06	3378 $\pm$ 128 (31.4)	-180 $\pm$ 142 (26.4)	185 $\pm$ 212 (34.6)	0.096	-574 $\pm$ 140 (29.5)	591 $\pm$ 164 (24.0)	$\leq$ 0.001***
Aomori	40.82	3107 $\pm$ 110 (29.3)	-167 $\pm$ 157 (31.6)	172 $\pm$ 150 (26.6)	$\leq$ 0.05*	-599 $\pm$ 84 (19.7)	617 $\pm$ 142 (22.2)	$\leq$ 0.001***
Sendai	38.26	3501 $\pm$ 118 (28.1)	-213 $\pm$ 175 (31.4)	219 $\pm$ 153 (24.0)	$\leq$ 0.05*	-544 $\pm$ 129 (25.9)	560 $\pm$ 150 (21.4)	$\leq$ 0.001***
Tokyo	35.69	4986 $\pm$ 173 (28.8)	-350 $\pm$ 256 (32.6)	360 $\pm$ 220 (24.0)	$\leq$ 0.01**	-983 $\pm$ 141 (20.8)	1012 $\pm$ 208 (20.2)	$\leq$ 0.001***
Yokohama	35.44	5610 $\pm$ 197 (29.2)	-569 $\pm$ 232 (27.2)	586 $\pm$ 291 (27.3)	$\leq$ 0.001***	-1110 $\pm$ 153 (20.1)	1142 $\pm$ 244 (21.0)	$\leq$ 0.001***
Kyoto	35.01	5419 $\pm$ 162 (24.8)	-420 $\pm$ 212 (25.1)	432 $\pm$ 225 (22.4)	$\leq$ 0.001***	-917 $\pm$ 132 (17.3)	944 $\pm$ 194 (17.8)	$\leq$ 0.001***
Tokushima	34.07	4852 $\pm$ 132 (22.6)	-317 $\pm$ 207 (27.0)	326 $\pm$ 146 (16.5)	$\leq$ 0.001***	-872 $\pm$ 100 (14.8)	897 $\pm$ 120 (12.2)	$\leq$ 0.001***
Fukuoka	33.58	6012 $\pm$ 198 (27.3)	-569 $\pm$ 282 (30.6)	586 $\pm$ 242 (21.4)	$\leq$ 0.001***	-1259 $\pm$ 135 (16.8)	1296 $\pm$ 209 (16.6)	$\leq$ 0.001***
Kochi	33.57	7264 $\pm$ 177 (20.2)	-397 $\pm$ 222 (19.1)	409 $\pm$ 261 (19.8)	$\leq$ 0.001***	-1082 $\pm$ 155 (14.8)	1114 $\pm$ 176 (12.2)	$\leq$ 0.001***
Oita	33.24	4756 $\pm$ 140 (24.5)	-439 $\pm$ 166 (22.8)	452 $\pm$ 203 (22.7)	$\leq$ 0.001***	-884 $\pm$ 99 (15.1)	910 $\pm$ 151 (15.5)	$\leq$ 0.001***
Miyazaki	31.94	8432 $\pm$ 182 (17.9)	-289 $\pm$ 280 (20.4)	297 $\pm$ 224 (14.9)	$\leq$ 0.05*	-1167 $\pm$ 142 (11.6)	1201 $\pm$ 176 (10.6)	$\leq$ 0.001***
Kagoshima	31.56	8024 $\pm$ 201 (20.8)	-646 $\pm$ 273 (21.9)	665 $\pm$ 251 (16.8)	$\leq$ 0.001***	-1319 $\pm$ 155 (13.6)	1358 $\pm$ 182 (11.3)	$\leq$ 0.001***

\*:  $p \leq 0.05$ ; \*\*:  $p \leq 0.01$ ; \*\*\*:  $p \leq 0.001$

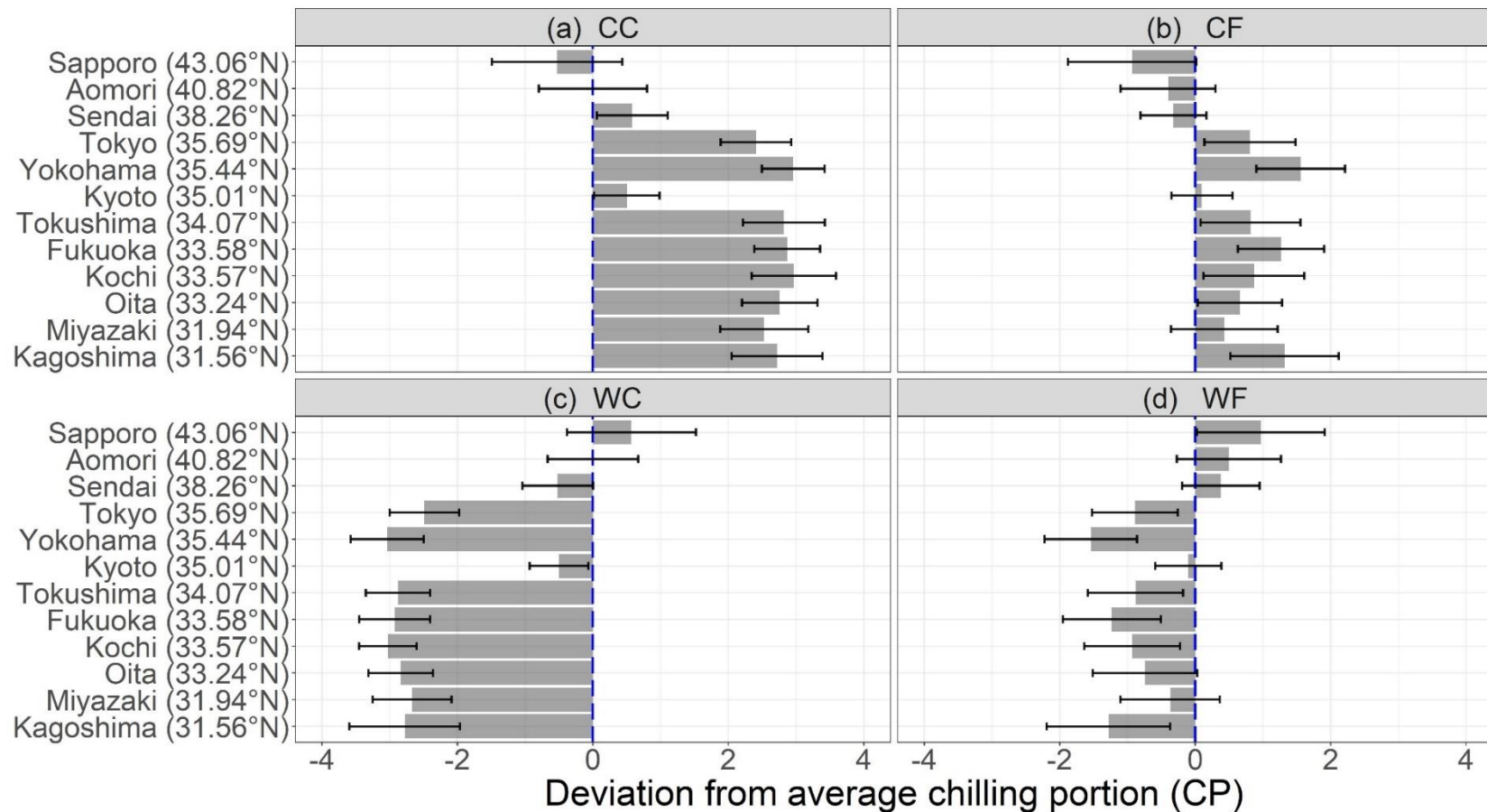


Figure SB2.1. The deviation of Yoshino cherry's chilling requirements (chilling portion, CP) from the average chilling requirement of four chilling-forcing combinations over the entire study period from 1953 to 2021 in different latitudes and locations. (a) CC: cold chilling, (b) CF: cold forcing, (c) WC: warm chilling, (d) WF: warm forcing. Positive values mean more CP than the average, and negative values indicate less CP than average. The error bars is standard errors.

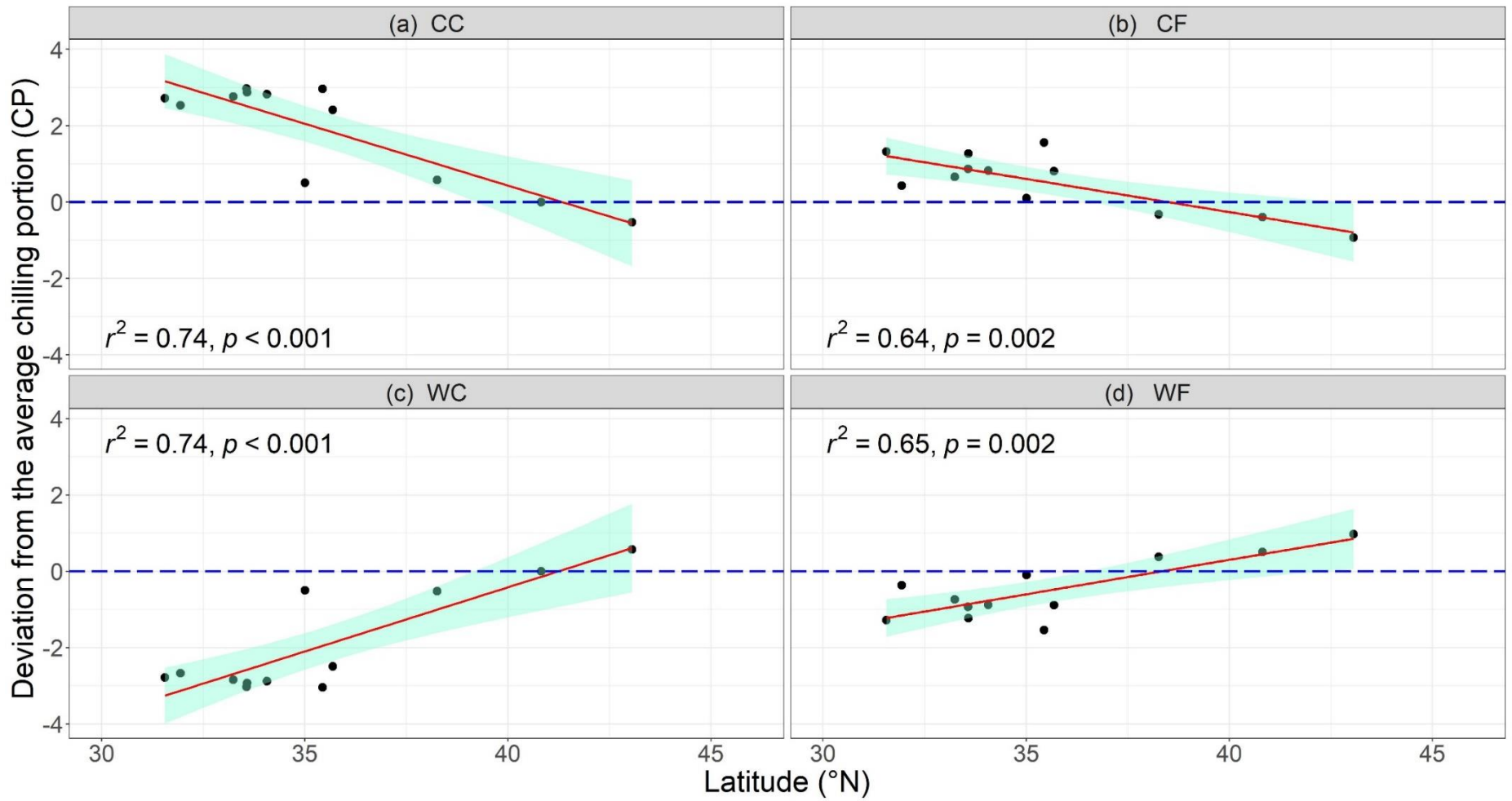


Figure SB2.2. A simple linear regression of the deviation of Yoshino cherry's chilling requirements (chill portion, CP) from the average chilling requirements in different chilling and forcing temperatures across the latitudinal gradient over the entire study period from 1953 to 2021. (a) CC: cold chilling, (b) CF: cold forcing, (c) WC: warm chilling, (d) WF: warm forcing. Blue shadows are 95% confidence intervals.

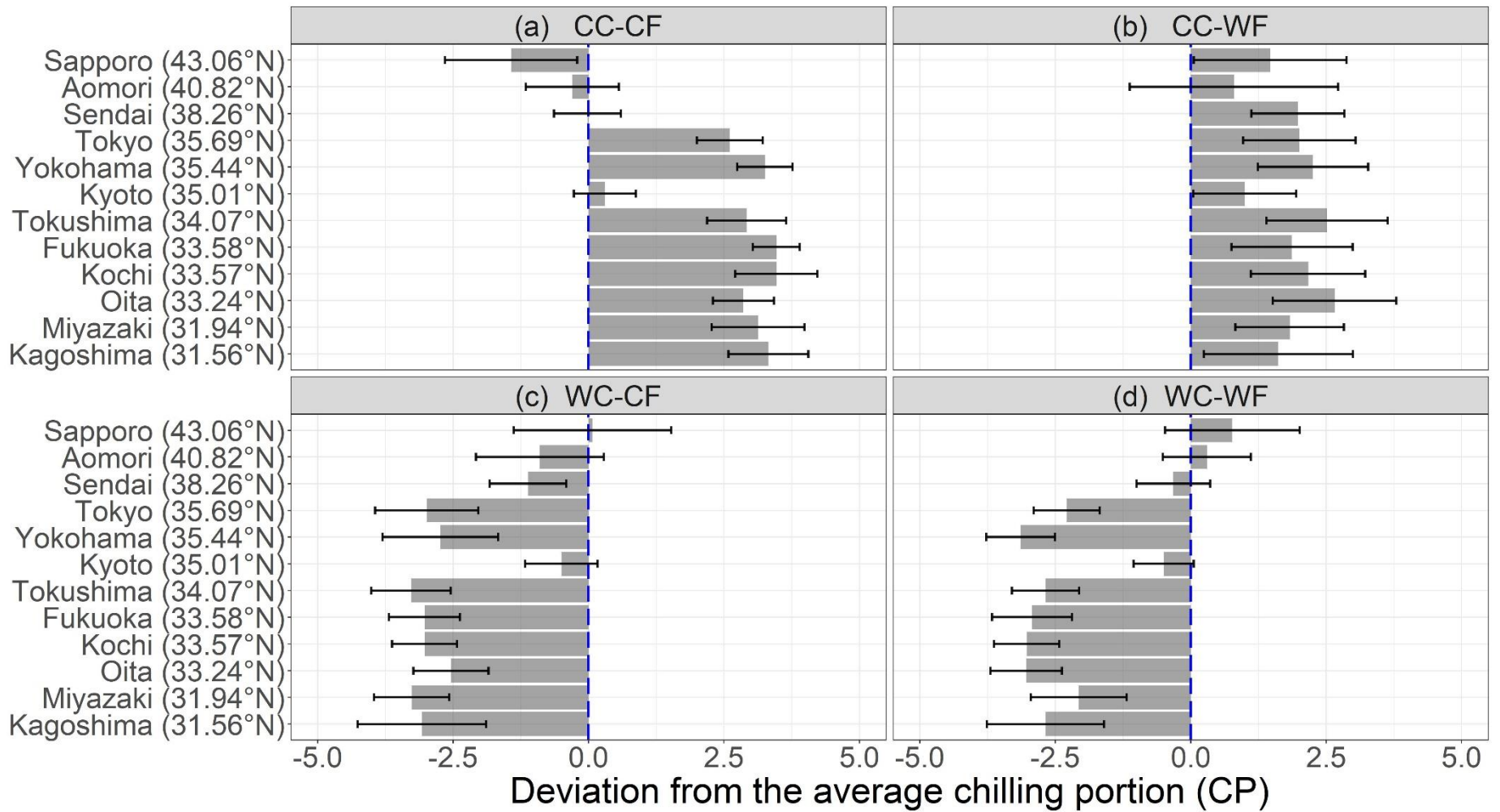


Figure SB2.3. The deviation of Yoshino cherry’s chilling requirement (chilling portion, CP) from the average chilling requirement of four chilling-forcing combinations over the entire study period from 1953 to 2021 in different latitudes and locations. (a) Cold chilling-cold forcing (CC-CF); (b) cold chilling-warm forcing (CC-WF); (c) warm chilling-cold forcing (WC-CF); (d) warm chilling-warm forcing (WC-WF). Positive values mean more CP than average, and negative values indicate less CP than average. The error bars are standard errors.

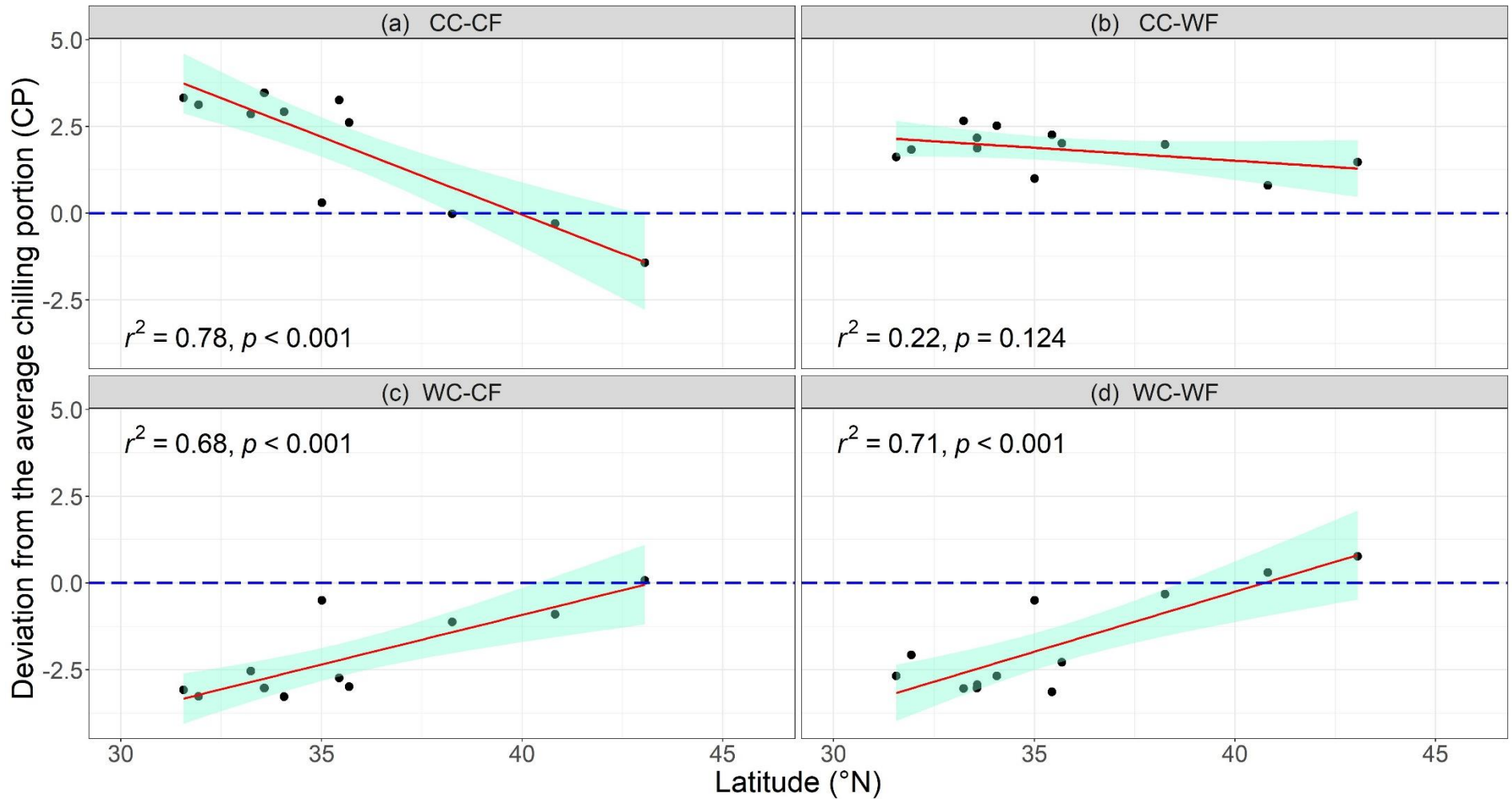


Figure SB2.4. A simple linear regression of the deviation of Yoshino cherry's chilling requirements (chill portion, CP) from the average chilling requirements in four different chilling-forcing combinations across the latitudinal gradient over the entire study period from 1953 to 2021. (a) cold chilling-cold forcing (CC-CF); (b) cold chilling-warm forcing (CC-WF); (c) warm chilling-cold forcing (WC-CF); and (d) warm chilling-warm forcing (WC-WF). Blue shadows are 95% confidence intervals.

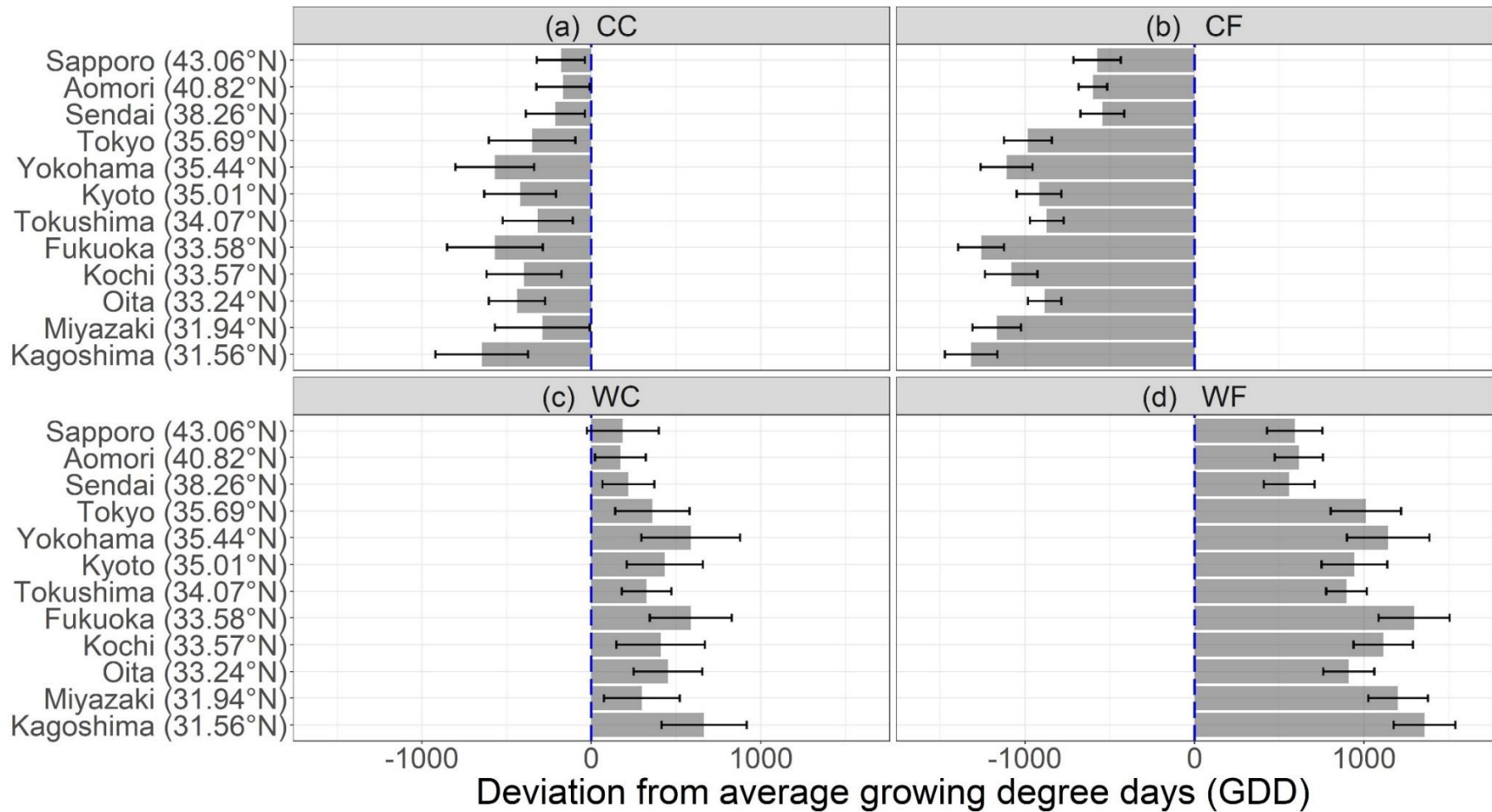


Figure SB2.5. The deviation of Yoshino cherry's forcing requirements (growing degree day, GDD) from the average forcing requirement of four chilling-forcing combinations over the entire study period from 1953 to 2021 in different latitudes and locations. (a) CC: cold chilling, (b) CF: cold forcing, (c) WC: warm chilling, (d) WF: warm forcing. Positive values mean more GDD than the average, and negative values indicate less GDD than average. The error bars is standard errors.

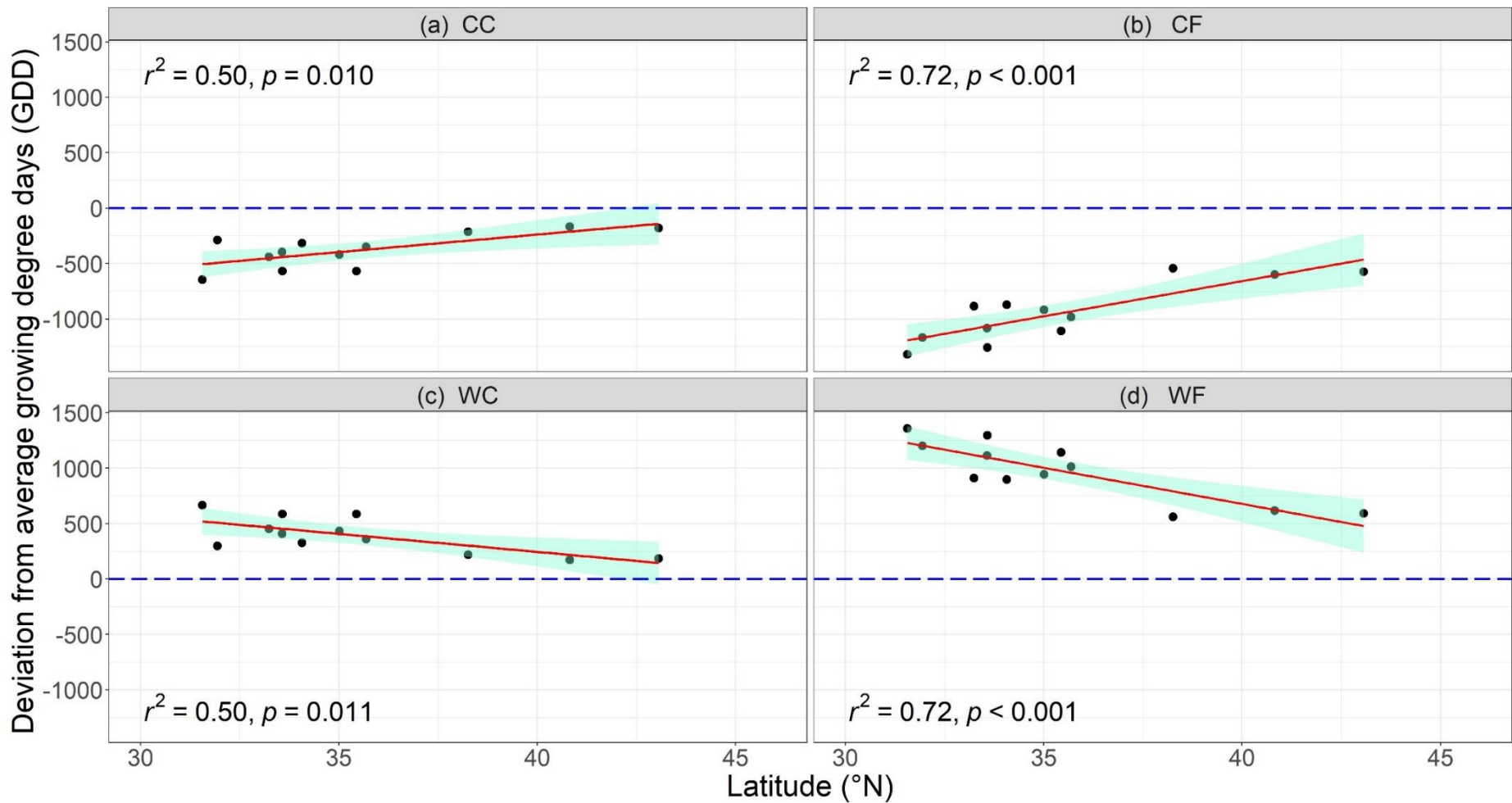


Figure SB2.6. A simple linear regression of the deviation of Yoshino cherry's forcing requirements (growing degree day, GDD) from the average chilling requirements in different chilling and forcing temperatures across the latitudinal gradient over the entire study period from 1953 to 2021. (a) CC: cold chilling, (b) CF: cold forcing, (c) WC: warm chilling, (d) WF: warm forcing. Blue shadows are 95% confidence intervals.

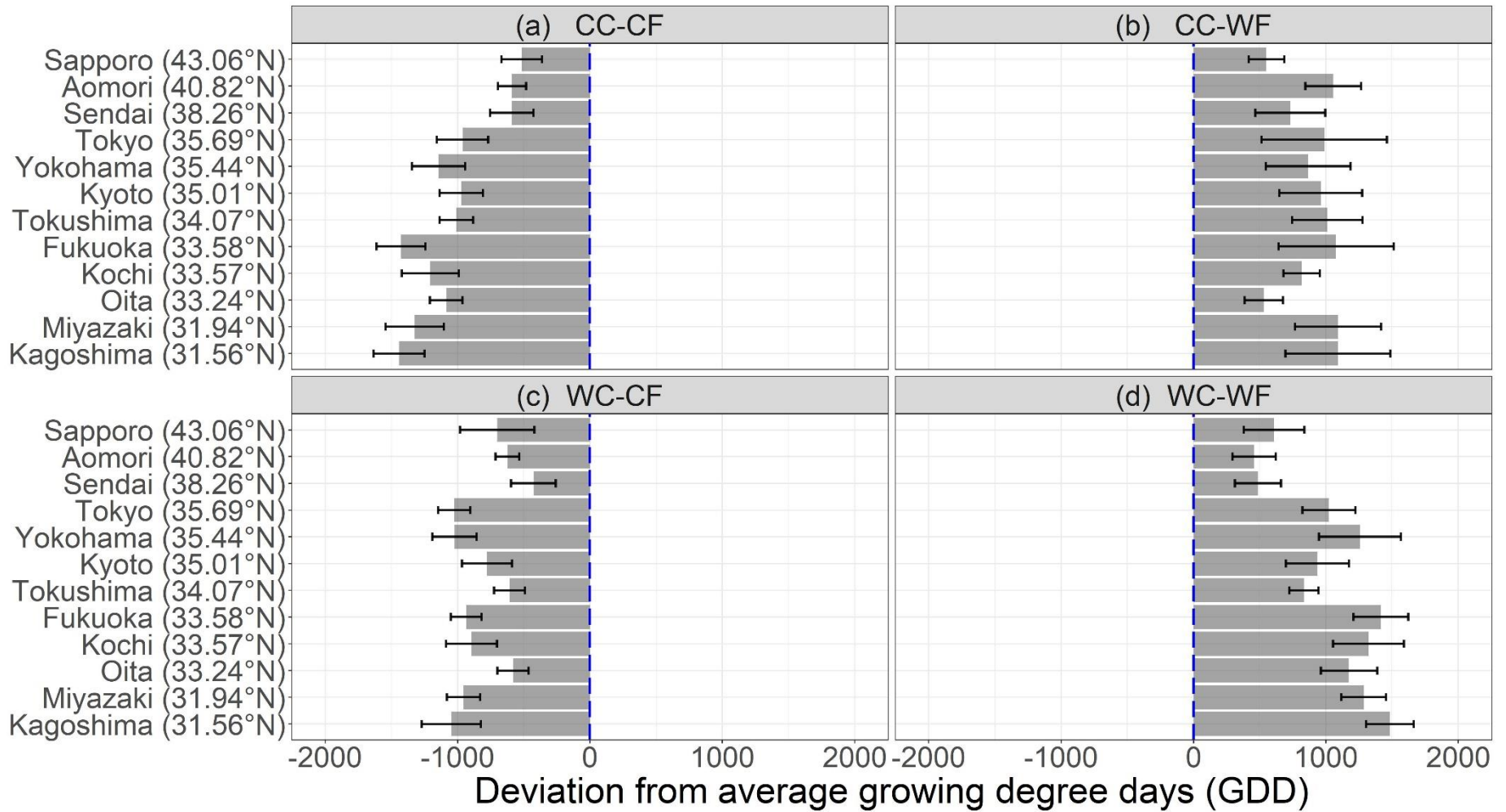


Figure SB2.7. The deviation of Yoshino cherry's forcing requirement (growing degree day, GDD) from the average forcing requirement of four chilling-forcing combinations over the entire study period from 1953 to 2021 in different latitudes and locations. (a) Cold chilling-cold forcing (CC-CF); (b) cold chilling-warm forcing (CC-WF); (c) warm chilling-cold forcing (WC-CF); (d) warm chilling-warm forcing (WC-WF). Positive values mean more GDD than average, and negative values indicate less GDD than average. The error bars are standard errors.

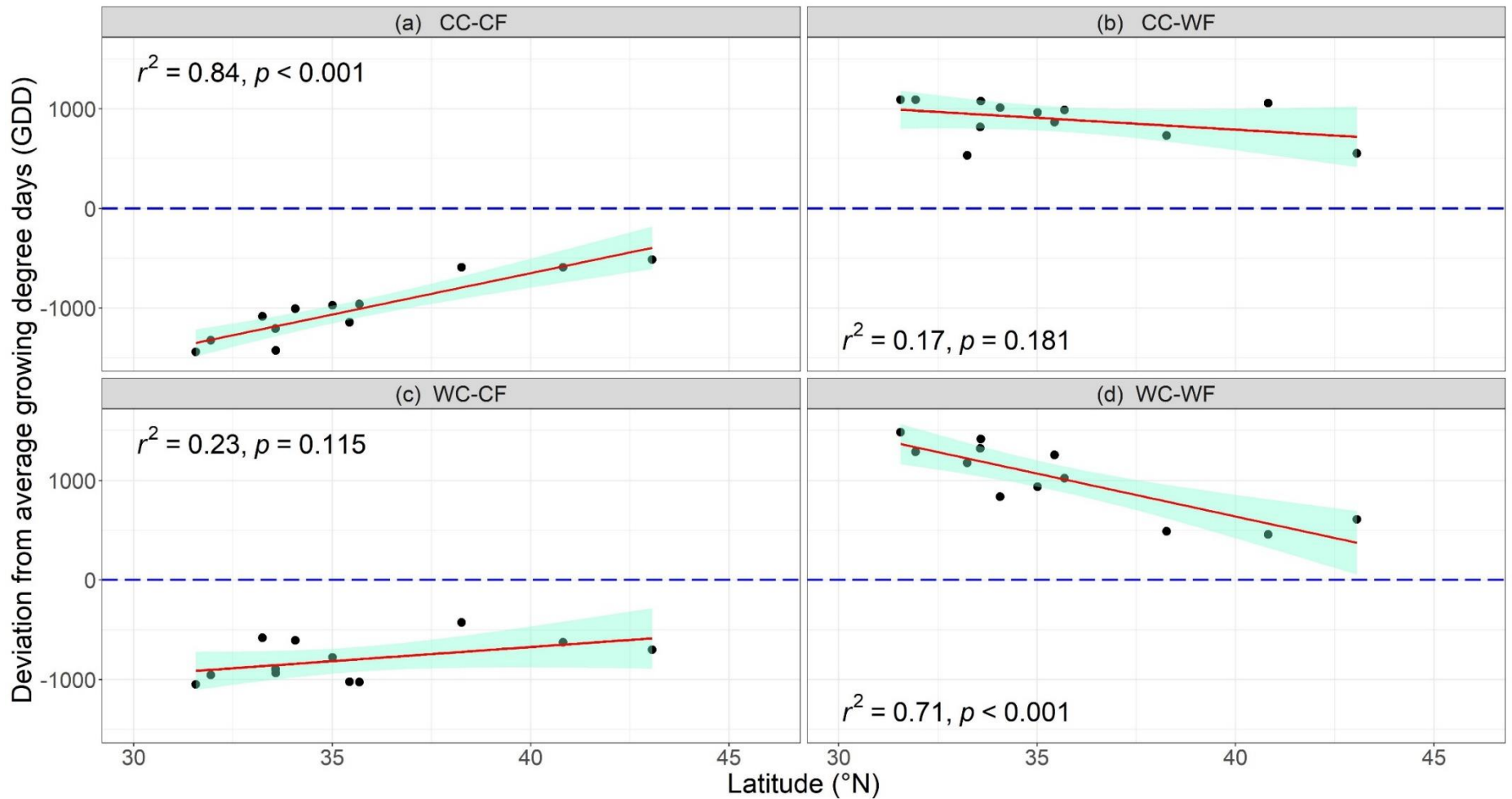


Figure SB2.8. A simple linear regression of the deviation of Yoshino cherry's forcing requirements (growing degree day, GDD) from the average forcing requirements in four different chilling-forcing combinations across the latitudinal gradient over the entire study period from 1953 to 2021. (a) cold chilling-cold forcing (CC-CF); (b) cold chilling-warm forcing (CC-WF); (c) warm chilling-cold forcing (WC-CF); and (d) warm chilling-warm forcing (WC-WF). Blue shadows are 95% confidence intervals.

## Chapter 3

### Temperature dependence of pollen germination and tube growth of three conifers in Washington State, USA

#### Authors:

Hsin-Wu Hsu<sup>1</sup>,

ORCID: 0000-0002-6347-4540

E-mail address: [hwhsu@uw.edu](mailto:hwhsu@uw.edu)

Soo-Hyung Kim<sup>1\*</sup>,

ORCID: 0000-0003-3879-4080

E-mail address: [soohkim@uw.edu](mailto:soohkim@uw.edu)

<sup>1</sup>School of Environmental and Forest Sciences, University of Washington, Seattle, WA 98195-4115, USA

\*Corresponding author

#### Abstract

- *Background and Aims:* Pollen germination and tube growth are essential processes for successful fertilization. They are among the most temperature-vulnerable stages and subsequently affect seed production and determine population persistence and species distribution under climate change. Our study aims to understand intra- and inter-specific variations in the temperature dependence of pollen germination and tube length growth and to explore how these variations differ for pollen from elevational gradients.
- *Methods:* We focused on three conifer species, *Pinus contorta*, *Picea engelmannii*, and *Pinus ponderosa*, with pollen collected from 350 to 2200m elevation in Washington

State, USA. We conducted pollen viability tests at temperatures from 5 to 40°C in 5°C intervals. After testing for four days, we took images of these samples under a microscope to monitor pollen germination percentage (GP) and tube length (TL). We evaluated Gamma and Gaussian functions for their ability to describe the temperature dependence of GP and TL and estimated key parameters, including the optimal temperature for GP ( $T_{opt\_GP}$ ) and TL ( $T_{opt\_TL}$ ).

- *Key Results:* Results showed that pollen from different species and different elevations within a species have different GP, TL,  $T_{opt\_GP}$ , and  $T_{opt\_TL}$ . The population with a higher  $T_{opt\_GP}$  would also have a higher  $T_{opt\_TL}$ , while  $T_{opt\_TL}$  was generally higher than  $T_{opt\_GP}$ . The variability in GP increased at extreme temperatures, whereas the variability in TL was greatest near  $T_{opt\_TL}$ .
- *Conclusions:* Our study demonstrates the temperature dependences of three conifers across a wide range of temperatures. Pollen germination and tube growth are highly sensitive to temperature conditions and vary among species and elevations. Pollen germination traits of different species and different elevations within a species are correlated with their ecological niche, and  $T_{opt\_GP}$  and  $T_{opt\_TL}$  have a positive relationship. Our findings can provide valuable insights to advance our understanding of how conifer pollen responds to warming.

**Keywords:** Conifer, *Pinus contorta*, *Picea engelmannii*, *Pinus ponderosa*, Pollen germination, Pollen tube length, Temperature dependence, Intraspecific variation, Elevational gradient, Climate change

### 3.1 Introduction

Temperature is a major climatic factor limiting plant species' geographical distributions (Rosbakh & Poschlod, 2016) and is expected to increase globally from 1.4°C (low GHG emission scenario) to 4.4°C (very high GHG emission scenario) with frequent temperature extremes by

the end of this century (Calvin et al., 2023). Reproductive phases, e.g., pollen germination, pollen tube growth, and fruit set, are among the most temperature-sensitive and vulnerable stages (Bykova et al., 2012; Hedhly et al., 2009; Kakani et al., 2005; Zinn et al., 2010). However, species distribution models rarely consider temperature-induced reproductive failures (Bykova et al., 2012). Temperature stresses on the pollen of crops and fruit trees have been studied frequently (e.g., Hedhly et al., 2004, 2005b; Kakani et al., 2002; Liu et al., 2023) due to the risk to food security. Nonetheless, very little is known about the susceptibility of conifer pollen to warm temperatures and whether they can adapt or acclimate to heat stress within populations (Flores-Rentería et al., 2018). Most studies of conifer pollen (Owens et al., 1998; Owens & Simpson, 1986), including *Pinus spp.* (e.g., Moody & Jett, 1990; Parantainen & Pasonen, 2004; Parantainen & Pulkkinen, 2002; Siregar & Sweet, 2000), *Pseudotsuga menziesii* (Mirb.) Franco (e.g., Dumont-BéBoux & Von Aderkas, 1997; Owens et al., 1981; Webber, 1987; Webber & Bonnet-Masimbert, 1993), *Picea engelmannii* Parry ex Engelm. (Owens et al., 1987; Webber, 1995), and *Thuja plicata* Donn ex D. Don (Colangeli & Owens, 1990), were conducted decades ago for seed production purposes in seed orchards, tree improvement, and breeding programs (Owens et al., 1998). There were few studies investigating how conifer pollen responds to warming or whether the warming would affect reproduction and the species' persistence in the future at that time. When high-temperature stress is applied separately on male and female gametes before pollination, it is frequently observed that pollen is often the most vulnerable link in the reproductive cycle (Zinn et al., 2010). The ability and speed of pollen germination and the rate of pollen tube growth under different temperatures are traits that can be selected for increasing the probability of successful fertilization by pollen from a particular donor (Pasonen et al., 1999, 2000, 2001, 2002; Skogsmyr & Lankinen, 2002) and consequently, shape the genetic structure and adaptation of next generations. Therefore, determining the temperature dependence of conifer pollen germination and pollen tube growth from different conifer species and populations is essential to evaluate species' response to temperature for predicting and mitigating the impacts of climate change on plant populations and ecosystems, but is rarely studied in conifers.

The reproductive phase in flowering plants is highly sensitive to hot or cold temperatures. It is well documented for numerous crops (e.g., tomato, soybean, corn, groundnut, etc.) and fruit trees (e.g., cherry, citrus, longan, mango, etc.) and was reviewed in the past (Hedhly et al., 2009; Thakur et al., 2010; Zinn et al., 2010). Some studies in high-mountain flowering plants (Rosbakh & Poschlod, 2016; Steinacher & Wagner, 2012) and temperate or tropical deciduous trees (Luza et al., 1987; Pasonen et al., 2000) also showed that pollen germination and tube growth has diverse optimal, cold- and heat-limited temperatures. The temperature has a clearly different effect on pollen germination and the rate of pollen tube growth in flowering plants (Hedhly et al., 2004; Kakani et al., 2002; McKEE, 1998). While warmer temperatures decreased germination percentage, they accelerated the growth rate of pollen tubes, and the different optimal temperatures for pollen germination and tube growth suggest an independent control (Hedhly et al., 2004). Conifer pollen and pollen tubes exhibit numerous distinctive traits absent in flowering plants; examples include reduced rate and extended period of growth, extremely delayed sperm formation, no cytokinesis following sperm formation, a pollen tube wall made up primarily of cellulose, and distinct cytoskeletal control and organelle zonation (Fernando et al., 2005). Little is known about whether these trait differences will result in different temperature response rates or directions in conifers. Thus, the study of pollen germination and tube development in conifers not only provides valuable insights into a more rudimentary form of sexual reproduction (Fernando et al., 2005) but also enhances our understanding of this crucial phase in the reproductive cycle of conifers. Our understanding of pollination in conifers has advanced rapidly in the past two decades (Breygina et al., 2021; Dumont-BéBoux & Von Aderkas, 1997; Fernando et al., 1997, 2005; Owens et al., 1981, 1987, 1998) but it still falls behind our knowledge of this process in flowering plants. Some conifer pollen studies have shown that different species or the same species from different habitats have very different optimal germination temperatures; for example, seven *Pinus* species from South Africa have optimal germination at 32°C (Nel et al., 2005), while Scots pine from Finland has germination percentage from 62 to 92% at 20°C (Parantainen & Pulkkinen, 2002). In addition, the pollen germination and tube growth of Scots pine from northern populations is greater at higher temperatures, whereas pollen from southern populations is unaffected (Varis et al., 2011).

These inter- and intra-specific variations may result in different responses in successful fertilization and consequently, seed production under climate change since pollen germination and tube growth are highly temperature-dependent. Some studies have shown that pollen germination test in vitro is a good predictor of total seed and percent filled seed in loblolly pine (Moody & Jett, 1990) and Douglas-fir (Webber & Bonnet-Masimbert, 1993). Therefore, pollen viability represents a good way to study temperature-induced reproductive failure and its potential effects on population dynamics and species distribution (Rosbakh & Poschlod, 2016).

Temperature ranges and optima for reproduction are known to vary among species and cultivars and reflect the adaptation of species to average temperature during the flowering period (Hedhly et al., 2004, 2005a; Pham et al., 2015). It has been suggested that pollen of species from habitats with a higher mean annual temperature are adapted to germinate and grow under relatively high temperatures (Pasonen et al., 2000) and that pollen of crop species and cultivars flowering under relatively high temperatures germinates and grows tubes at relatively high temperatures (Kakani et al., 2005; Luza et al., 1987). A study in the Bavarian Alps along an elevational gradient found a strong positive relationship between temperature conditions at pollen collection sites and the minimum temperature for both pollen germination and pollen tube growth and a significant correlation between the maximum temperature of pollen tube growth and temperature of the flowering month (Rosbakh & Poschlod, 2016). Some high-mountain plants also showed remarkably flexible pollen performance over a wide temperature range (Steinacher & Wagner, 2012). Another study showed that Scots pine along a south-north gradient in Finland have variations in pollen viability both among and within latitudes and also among different temperature treatments (Parantainen & Pulkkinen, 2002). All these results indicate consistently strong correlation between habitat temperature and the temperature requirements of the pollen germination and tube growth and are, potentially, important contributors to the climatic restriction of plant species distributions, including the restriction by low temperatures (Rosbakh & Poschlod, 2016). Improved knowledge of the thermal requirement in pollen germination, a precursor to seed production, could enhance our understanding of species distributions along climatic gradients and our ability to predict how climate change might affect plant community composition (Rosbakh & Poschlod, 2016).

Environmental conditions vary along latitudinal and elevational gradients. In general, temperature decreases from low to high elevation and latitude, allowing for the elevational and latitudinal gradients to be used as proxies for studying plant species' responses to temperature (Rosbakh & Poschlod, 2016; Wu et al., 2019). Our primary goal is to determine the temperature dependence of pollen germination and tube growth in three conifers with high economic and ecological significance and unique niches vulnerable to climate change due to various complex and interacting reasons. We aim to explore the research questions of (1) how temperature dependence of pollen germination and tube growth varies among pollen collected from different elevations, (2) whether the optimal temperatures for pollen germination ( $T_{opt\_GP}$ ) and tube growth ( $T_{opt\_TL}$ ) are correlated to pollen collection elevations or to the temperatures when pollen sheds, and (3) if and how the  $T_{opt\_GP}$  and  $T_{opt\_TL}$  are related. Our hypothesis for the first research question ( $H_1$ ) is that the pollen from higher elevations (cooler sites) will have higher germination percentages and longer tube lengths at lower temperatures and vice versa because the germination responses to temperature may vary among populations across species' geographical distribution since they have acclimated or adapted to local climatic conditions or habitats (Chamorro et al., 2018). For the second research question, the hypothesis ( $H_2$ ) is that the  $T_{opt\_GP}$  is correlated to both pollen collection elevations and temperatures when pollen sheds because they have acclimated or adapted to the local environments. Our hypothesis for the third research question is that the  $T_{opt\_GP}$  and  $T_{opt\_TL}$  are different and correlated because ( $H_{3a}$ ) the mechanisms involved are different, ( $H_{3b}$ ) pollen tube grows later in the season (i.e., warmer) than pollen germinates, and ( $H_{3c}$ ) they have also acclimated or adapted to the local environments. Temperature is an essential factor controlling plant reproduction. Climate change could affect the pollination process depending on prevailing conditions, species distribution, and environmental tolerance, but is rarely considered in species distribution models. We anticipate the results could advance our understanding of how conifer pollen responds to warming and provide valuable information for enhancing the projection of conifer species' distribution models under climate change.

## 3.2 Materials and Methods

### 3.2.1 Species and pollen collection sites

We collected pollen of three conifer species, Lodgepole pine (*Pinus contorta* Dougl. ex. Loud.; PICO), Engelmann spruce (*Picea engelmannii* Parry ex Engelm.; PIEN), and Ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.; PIPO), at Tye Mountain (350 to 2000m; Entiat, Washington, USA) and Slate Peak (1500 to 2200m; Mazama, Washington, USA) in Washington State (Figure 3.1). Nine Pendant Temperature/Light Data Loggers UA-002-64 (Onset HOBO, Bourne, Massachusetts, USA) were deployed to record hourly mean air temperatures at Tye Mountain along the elevational gradient and seven loggers at Slate Peak along the elevational gradient from October 2020 to June 2021 (Figure 3.1). The recorded spring mean temperature (April, May, and June) ranges at the pollen collection sites for each species are 3.4 to 6.1°C (PICO), 1.7 to 7.4°C (PIEN), and 7.1 to 13.7°C (PIPO) (Table S3.1).

Lodgepole pine is a species with a broad ecological amplitude. It grows under a wide variety of climatic conditions. Pollen generally matures in mid-May to mid-July and relates to elevation and climate (Burns & Honkala, 1990). Engelmann spruce grows in a humid climate with long, cold winters and short, cool summers. It occupies one of the highest and coldest forest environments in the western United States. Pollen is shed in late May and early June at low elevations and from mid-June to early July at high elevations (Burns & Honkala, 1990). Ponderosa pine is one of the most widely distributed pines in western North America. Depending on locations, pollen is shed in mid-April to late June (Burns & Honkala, 1990). Ponderosa pine and Engelmann spruce are two species that occupy opposite ecological niches; that is, Ponderosa pine is often found in hot and dry environments at low elevations, and Engelmann spruce is found in cold and wet environments at high elevations on mountains. These three species occupy a wide range of climate niches in Washington State and the western United States (Table S3.1).

We collected matured male cones before the scales opened and shed pollen. PIPO pollen was collected from May 7 to June 5, 2021, at elevations from 350 to 1500m. PICO pollen was collected from June 5 to 21, 2021, at elevations from 1100 to 2000m. As PIEN matures late

around June at our sites, their pollens were collected from June 6 to 30, 2021, at elevations ranging from 1500 to 2200m. After the pollen collection, we placed male cones at room temperature drying for five days. We then collected the pollen grains and moved them to the fridge (4°C) prior to the viability tests.

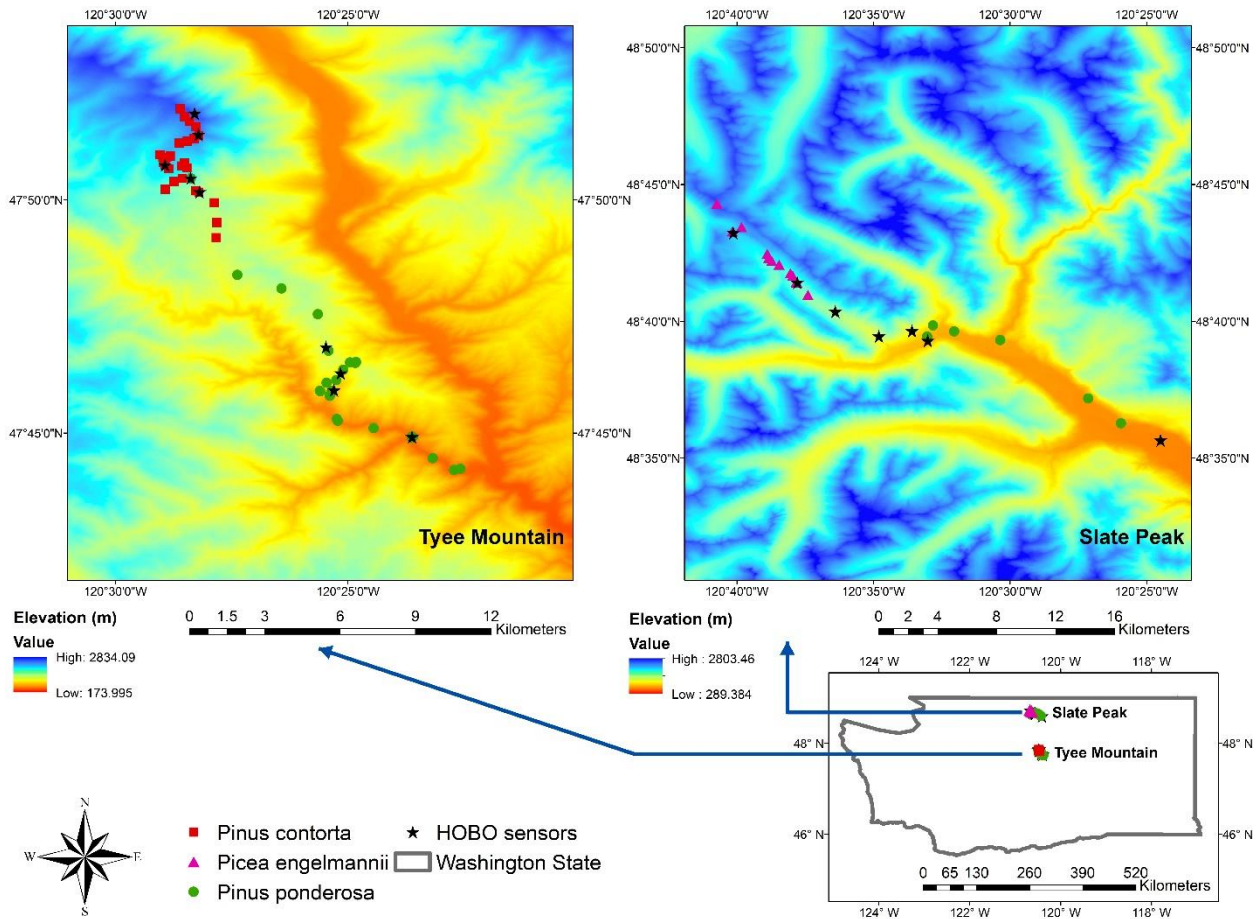


Figure 3.1 Temperature data and pollen of *Pinus contorta*, *Picea engelmannii*, and *Pinus ponderosa* were collected from the elevational gradients at Tye Mountain and Slate Peak in Washington State, USA.

### 3.2.2 Pollen germination test

A suspension culture method (Shivanna & Rangaswamy, 1992) was used for the pollen germination test. We first cultured pollen with the germination medium of Brewbaker and Kwack (1963), which was autoclaved and supplemented with thiamine, riboflavin, and ascorbic acid (Varis et al., 2011; Table S3.2). The vials were also autoclaved before the test, and 5 ml of culture media and 25 mg of pollen grains were added to each vial. They were then placed on CO-Z orbital shakers (80 revolutions per minute; Amazon, Seattle, USA) in G-1000 growth chambers (Conviron, Winnipeg, Canada) with temperatures from 5 to 40°C with an interval of 5°C for four days without light. At the end of tests, 3 ml of 0.1% (w/v) aniline blue-lactic acid-glycerol-water solution was added to each vial to arrest pollen tube growth and stain the tubes blue (Parantainen & Pasonen, 2004; Parantainen & Pulkkinen, 2002).

Pollen from multiple elevations (23 for PICO, 12 for PIEN, and 30 for PIPO) were used, and eight temperature treatments (5 to 40°C in 5°C intervals) for each species were applied in the test. Three test rounds (replicates) were conducted in different chambers. In each test round, two subsamples were collected in each vial and considered to be repeat observations. The total number of samples in each species is shown in Table S3.3. Each subsample was placed on a micro slide and covered with a cover glass. We then took images of these samples under the Trinocular Stereo Microscope SM-2T-LED and 10MP USB 3.0 Color CMOS C-Mount Microscope Camera MU1003 (AmScope, Irvine, California, USA) with the magnification of x16 to x40, depending on the species, to monitor pollen germination and pollen tube growth. The germination percentage was determined by counting pollen grains from the top left to the bottom right of each image until 200 pollen grains were selected per sample. Pollen grain was considered germinated when the length of its tube was more than the diameter of the pollen grain (Shivanna & Rangaswamy, 1992; Varis et al., 2011). The tube lengths of the first 20 germinated pollen grains from the 200 counts per subsample were selected and measured with the help of AmScope software (version x64, 4.11.21973.20230107, AmScope, Irvine, California, USA). If there were less than 20 pollen grains germinated, we measured all germinated pollen tube lengths. Only the longest pollen tube was measured if the tube had branches (Parantainen & Pasonen, 2004; Parantainen & Pulkkinen, 2002). We used the raw tube length data in each

subsample for analysis. As for the germination percentage, subsamples were considered repeat observations, and the three test rounds were true replicates.

### 3.2.3 Modeling pollen germination percentage and tube length growth

Bell-shaped or peak functions have been widely applied in agricultural science to describe the rate of biological processes as a function of temperature (Archontoulis & Miguez, 2015). We applied both the Gaussian (Eqn. 3.1) and Gamma function (Eqn. 3.2) to evaluate their ability to describe the temperature dependence of pollen germination germination percentage (GP) and tube length (TL). The Akaike Information Criterion (AIC) was used to assess the relative quality of Gaussian and Gamma functions in our study.

$$G = G_{max} \exp\{-0.5[(T - T_o)/b]^2\} \quad \text{Eqn. 3.1}$$

In Eqn. 3.1,  $G$  is the response variable (pollen germination percentage, unit: %, or tube length, unit:  $\mu\text{m}$ ),  $T$  is the explanatory variable (temperature, unit:  $^{\circ}\text{C}$ ),  $G_{max}$  is the maximum  $G$  value,  $T_o$  (optimal temperature, unit:  $^{\circ}\text{C}$ ) is the position of the peak ( $G_{max}$ ), and  $b$  is the coefficient controlling the width of the bell shape.

$$G = \alpha T^{\beta} \exp(-\theta T) \quad \text{Eqn. 3.2}$$

In Eqn. 3.2, the definitions of  $G$  and  $T$  are the same as in Eqn. 3.1, and  $\alpha$  (no unit) determines the overall scale or height of the curve,  $\beta$  (no unit) controls the shape of the curve, particularly the steepness of its rise, and  $\theta$  (no unit) affects the curve's position along the x-axis (horizontal shift) and influences the curve's decay rate. The GP and TL Gamma temperature response curves of three elevations (low, medium, and high) of each species close to the regression lines in Figure 3.4 were selected to visualize the change of temperature response curves across elevational gradients. Because the regression lines of GP and TL of each species are different in Figure 3.4, the low, medium, and high elevations close to the regression lines are slightly different.

The Michaelis-Menten equation is well-known and routinely applied to quantify the rate of a process dependent on the substrate (Archontoulis & Miguez, 2015). Since pine pollen grains

may accumulate “prepackaged” rRNA that is preprogrammed to synthesize all the proteins needed throughout the entire duration of the germination and tube growth process (Frankis, 1990) and higher spring mean temperatures at each site speed up the protein-synthetic process in each species, it is reasonable to model the  $T_{opt\_GP}$  and  $T_{opt\_TL}$  versus spring mean temperatures across species with a Michaelis-Menten function (Eqn. 3.3) to test  $H_2$ .

$$X_o = mT_s/(n + T_s) \quad \text{Eqn. 3.3}$$

In Eqn. 3.3,  $X_o$  is the response variable (optimal temperatures for each species and elevations, unit: °C),  $T_s$  is the explanatory variable (spring temperatures at different sites, unit: °C),  $m$  is the higher asymptote of  $X_o$  ( $T_s \rightarrow X_o$ , unit: °C), and  $n$  is the  $T_s$  value giving a response equal to  $m/2$  (unit: °C). We restricted the spring temperature to no greater than the optimal temperature ( $T_s \leq X_o$ ) in this equation. A summary of each variable and parameter in Eqn. 3.1, Eqn. 3.2, and Eqn. 3.3 are shown in Table 3.1.

Table 3.1 Variables and parameters used in the Gaussian, Gamma, and Michaelis-Menten functions and their descriptions.

Symbol	Unit	Description
<b>Gaussian function [Eqn. 3.1]</b>		
$G$	%	The response variable: germination percentage.
	$\mu\text{m}$	The response variable: tube length.
$T$	$^{\circ}\text{C}$	The explanatory variable: temperature.
$G_{max}$	%	Germination percentage: the maximum value for the expected response of $G$ .
	$\mu\text{m}$	Tube length: the maximum value for the expected response of $G$ .
$T_o$	$^{\circ}\text{C}$	The temperature at $G_{max}$ (optimal temperature).
$b$	-	Shape parameter controlling the width of the bell shape.
<b>Gamma function [Eqn. 3.2]</b>		
$G$	%	The response variable: germination percentage.
	$\mu\text{m}$	The response variable: tube length.
$T$	$^{\circ}\text{C}$	The explanatory variable: temperature.
$\alpha$	-	Shape parameter controlling the overall scale or height of the curve.
$\beta$	-	Shape parameter controlling the steepness of the curve's rise.
$\vartheta$	-	Shape parameter controlling the curve's position along the x-axis (horizontal shift) and its decay rate.
<b>Michaelis-Menten function [Eqn. 3.3]</b>		
$X_o$	$^{\circ}\text{C}$	The response variable: optimal temperatures for different species and elevations.
$T_s$	$^{\circ}\text{C}$	The explanatory variable: spring temperature at different sites.
$m$	$^{\circ}\text{C}$	The higher asymptote of $X_o$ ( $T_s \rightarrow X_o$ ).
$n$	$^{\circ}\text{C}$	The $T_s$ value giving a response equal to $m/2$

### 3.2.4 Statistical analysis

We used simple linear regression to fit (1) the observed GP and TL to different elevations within the same species and tested temperature and (2) the  $T_{opt\_GP}$  and  $T_{opt\_TL}$  to different elevations within the same species to test  $H_1$  and  $H_2$ . We also used a one-way analysis of variance (ANOVA) and Tukey's honest significance test (HSD) to test the  $T_{opt\_GP}$  and  $T_{opt\_TL}$  among species ( $H_2$ ). A t-test was used to compare the differences between each species'  $T_{opt\_GP}$  and  $T_{opt\_TL}$  ( $H_3$ ). A quadratic function was used to fit the coefficient of variation in each species

and temperature. The monthly mean temperature along the elevational gradients in Tye Mountain and Slake Peak were calculated. We then used simple linear regression to estimate the lapse rate of each month and site (Figures S3.1 and S3.2). All analyses were conducted in the R 4.1.2 programming language (R Core Team, 2021).

### 3.3 Results

#### 3.3.1 Modeling pollen GP and TL of each species at different temperatures across elevational gradients with Gaussian and Gamma functions.

The fitted Gaussian and Gamma curves of GP and TL of PICO, PIEN, and PIPO at different elevations are shown in Figures S3.3 and S3.4. The AICs showed that Gamma curves have lower values than Gaussian curves in the GP of PIEN, but there was not much difference between Gaussian and Gamma curves in the GP of PIEN and GP and TL of the rest species (Tables S3.4 and S5). The Gamma function provided additional flexibility than the Gaussian function to control the ascending rate before the optimal temperature and the decay rate after the optimal temperature and hence explained greater variability of GP and TL across elevations and species with lower AICs and was subsequently used to estimate  $T_{opt\_GP}$  and  $T_{opt\_TL}$  in our study. The GP and TL Gamma temperature response curves of three selected elevations (low, medium, and high) of each species showed clear differences at different elevations, especially in the GP of PICO (Figure 3.2a). It showed that the population from the high elevation has a lower  $T_{opt\_GP}$  and higher GP when the temperatures are below the  $T_{opt\_GP}$  (Figure 3.2a). On the contrary, the population from the low elevation has a higher  $T_{opt\_GP}$  and higher GP when the temperatures are above the  $T_{opt\_GP}$  (Figure 3.2a). We also showed GP and TL Gaussian temperature response curves of the same three elevations of Gamma curves in each species in Figure S3.5.

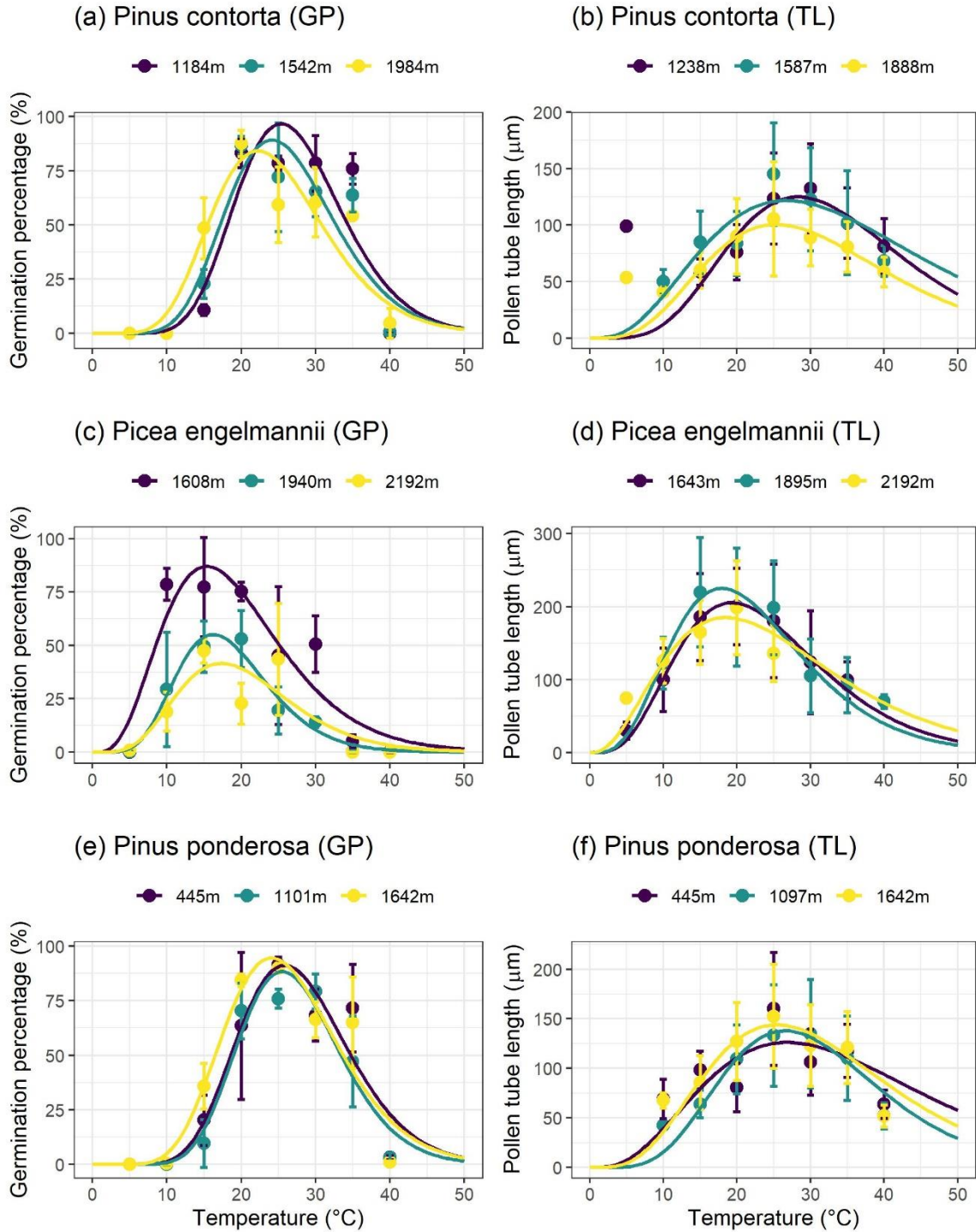


Figure 3.2 Gamma temperature response curves of pollen germination percentages (GP) and tube lengths (TL) in *Pinus contorta* (PICO; a and b), *Picea engelmannii* (PIEN; c and d), and *Pinus ponderosa* (PIPO; e and f) from three elevations (low, medium, and high) of each species that are close to the regression lines in Figure 3.4.

### 3.3.2 Intraspecific variations of pollen GP and TL in each temperature along the elevational gradient.

The slopes (unit: % change per °C) and  $p$ -values of each GP regression line across elevations in different species and temperatures are shown in Table 3.2. GP of PICO had a slope that is close to zero at 5°C ( $p=0.884$ ), positive slopes at 10, 15 ( $p<0.001$ ) and 20°C ( $p=0.002$ ), negative slopes at 25 ( $p=0.082$ ) and 30°C ( $p=0.005$ ), and slopes that are close to zero at 35 ( $p=0.108$ ) and 40°C ( $p=0.114$ ; Table 3.2 and Figure S3.6). TL of PICO showed that slopes are close to zero at 5 ( $p=0.059$ ) and 10°C ( $p=0.961$ ), positive slopes at 15 and 20°C ( $p<0.001$ ), and a negative slope at 25°C ( $p=0.006$ ), and slopes that are close to zero at 30 ( $p=0.575$ ), 35 ( $p=0.237$ ), and 40°C ( $p=0.158$ ; Table 3.2 and Figure S3.7). GP and TL of PIEN and PIPO also showed similar trends but different patterns due to their unique niche (Table 3.2 and Figures S3.8 to S3.11).

Table 3.2 The slopes (unit: % change per °C) and *p*-values of each regression line in pollen germination percentages (GP) and tube lengths (TL) of *Pinus contorta* (PICO), *Picea engelmannii* (PIEN), and *Pinus ponderosa* (PIPO) in each temperature along the elevational gradient.

Asterisks represented whether the slope is statistically different from zero at each temperature.

\*\*\*:  $p < 0.001$ ; \*\*:  $p < 0.01$ ; \*:  $p < 0.05$ .

Temperature (°C)	Species					
	PICO		PIEN		PIPO	
	<b>Pollen germination percentage (GP)</b>					
	Slope	<i>p</i> -value	Slope	<i>p</i> -value	Slope	<i>p</i> -value
5	0.000	0.884	-0.000	0.9	-0.000	0.499
10	0.003	<0.001***	-0.045	0.118	-0.0004	0.05*
15	0.080	<0.001***	-0.055	<0.001***	-0.001	0.849
20	0.016	0.002**	-0.050	0.015*	0.015	0.049*
25	-0.011	0.082	-0.041	0.04*	0.007	0.123
30	-0.021	0.005**	-0.008	0.772	-0.003	0.453
35	-0.013	0.108	-0.003	0.303	-0.010	0.112
40	0.003	0.114	-0.000	0.817	0.000	0.981
	<b>Pollen tube length (TL)</b>					
	Slope	<i>p</i> -value	Slope	<i>p</i> -value	Slope	<i>p</i> -value
5	-0.068	0.059	0.051	0.086	-0.055	0.006**
10	-0.000	0.961	0.009	0.273	-0.002	0.707
15	0.025	<0.001***	-0.041	0.009**	0.001	0.418
20	0.053	<0.001***	0.008	0.599	0.023	<0.001***
25	-0.014	0.006**	-0.084	<0.001***	-0.003	0.38
30	-0.003	0.575	-0.024	0.221	-0.021	<0.001***
35	-0.004	0.237	-0.012	0.696	-0.019	<0.001***
40	0.008	0.158	-	-	0.011	<0.001***

The variation of GP and TL showed opposite patterns (Figure 3.3). The coefficients of variation (CV) of GP of each species in all experimental rounds at different temperatures showed that variation increased when the temperatures shifted from optima toward upper or lower limits (Figures 3.3a, 3.3c, and 3.3e). The CV of PICO and PIPO also showed that variation was slightly higher at the lower-limit temperature than at the higher-limit temperature (Figures 3.3a and 3.3e). The CV of PIEN was higher at the higher-limit temperature than at the lower-limit temperature (Figure 3.3c). On the contrary, the CV of TL of each species in all experimental rounds at different temperatures showed that variations were the highest when the

temperatures were optimal and decreased when the temperatures shifted from optima toward upper or lower limits (Figures 3.3b, 3.3d, and 3.3f).

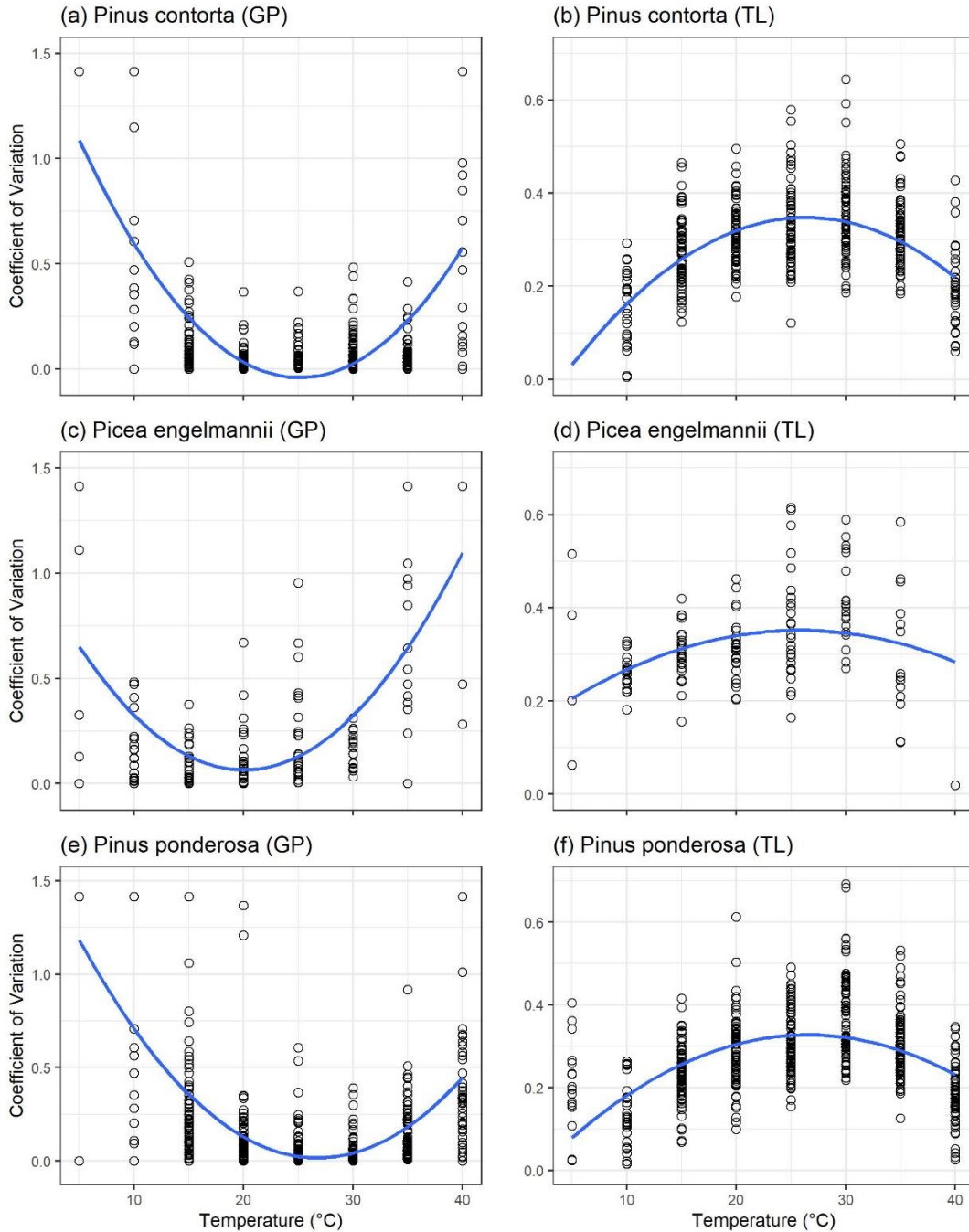


Figure 3.3 The coefficient of variations of pollen germination percentages (GP) and tube lengths (TL) in *Pinus contorta* (PICO; a and b), *Picea engelmannii* (PIEN; c and d), and *Pinus ponderosa* (PIPO; e and f) from different elevations at each temperature.

3.3.3 Inter- and intraspecific variations in  $T_{opt\_GP}$  and  $T_{opt\_TL}$  are correlated to pollen collection elevations or spring mean temperatures.

$T_{opt\_GP}$  and  $T_{opt\_TL}$  were significantly different between species (Tables 3.3 and S3.6), and PIEN ( $T_{opt\_GP}$ : 16.4°C,  $T_{opt\_TL}$ : 18.7°C) was the lowest, followed by PICO ( $T_{opt\_GP}$ : 23.6°C,  $T_{opt\_TL}$ : 26.5°C) and PIPO ( $T_{opt\_GP}$ : 26.2°C,  $T_{opt\_TL}$ : 26.8°C).  $T_{opt\_GP}$  and  $T_{opt\_TL}$  in PICO across the elevational gradient showed a significant descending trend ( $p < 0.001$  and  $p = 0.004$  in Figures 3.4a and 3.4b), but no significant trends were found along the elevational gradient in PIEN ( $p = 0.553$  and  $p = 0.886$ ) and PIPO ( $p = 0.649$  and  $p = 0.419$ ) (Figures 3.4c to 3.4f).

Table 3.3 The optimal temperature for pollen germination percentages ( $T_{opt\_GP}$ ) and tube lengths ( $T_{opt\_TL}$ ) of *Pinus contorta* (PICO), *Picea engelmannii* (PIEN), and *Pinus ponderosa* (PIPO). Different letters (a, b, c) indicate significant differences between species (row-wise comparisons) by using Tukey's honest significance test (HSD). \*\*\*:  $p < 0.001$ .

$T_{opt\_GP}$ (°C)			
PICO	PIEN	PIPO	p-value
23.6 ± 1.5 <sup>b</sup>	16.4 ± 1.3 <sup>c</sup>	26.2 ± 1.7 <sup>a</sup>	<0.001***
$T_{opt\_TL}$ (°C)			
26.5 ± 1.7 <sup>a</sup>	18.7 ± 2.0 <sup>b</sup>	26.8 ± 2.8 <sup>a</sup>	<0.001***

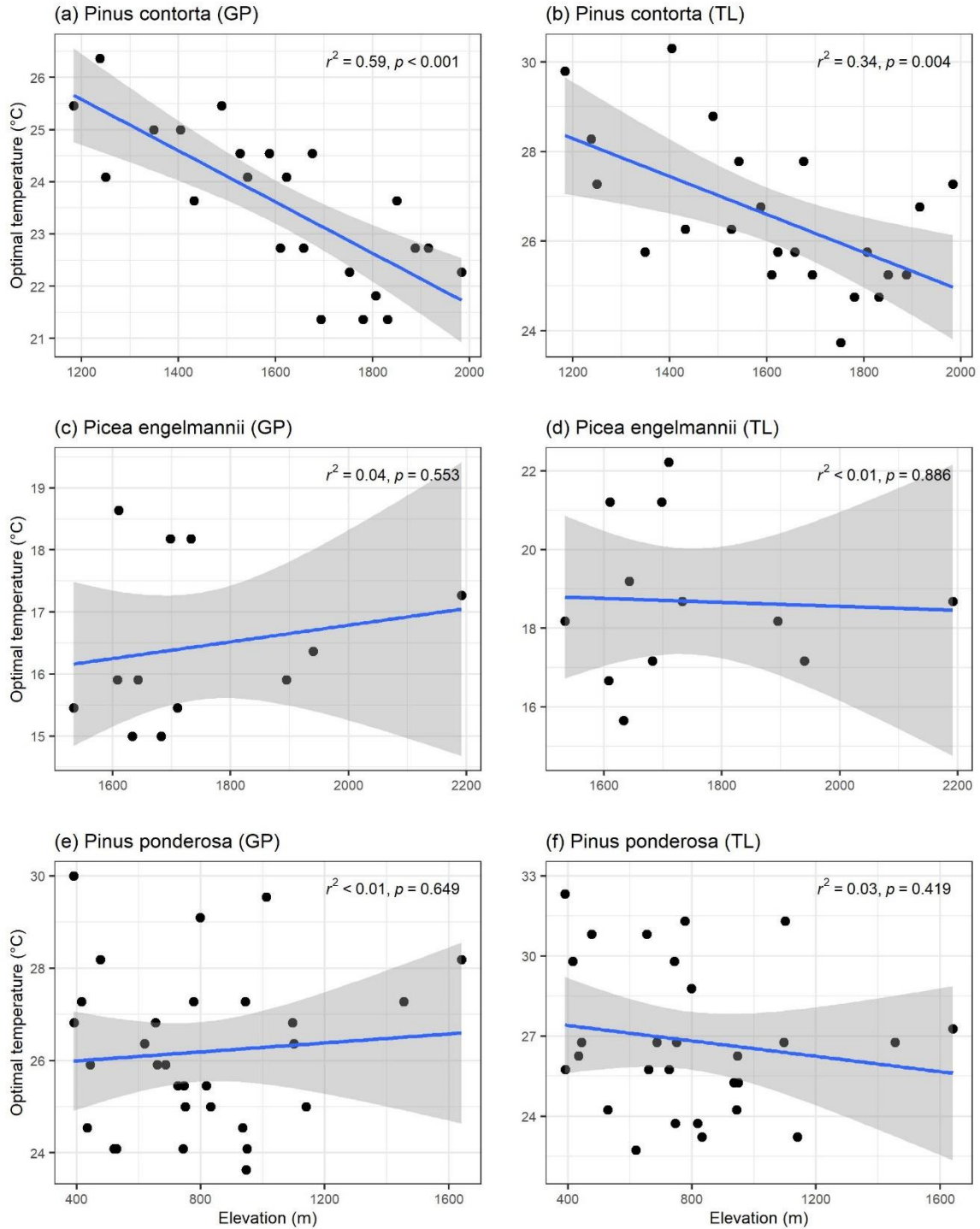


Figure 3.4 The regression lines of optimal temperatures of pollen germination percentages (GP) and tube lengths (TL) in *Pinus contorta* (PICO; a and b), *Picea engelmannii* (PIEN; c and d), and *Pinus ponderosa* (PIPO; e and f) across the elevational gradients. The shaded areas are 95% confidence intervals.

The  $T_{opt\_GP}$  of different species along the elevational gradient was also shown in Figure 3.5. PICO and PIEN from different sites had overlapping elevations but different  $T_{opt\_GP}$  ranges (Figure 3.5a). After converting the elevations to actual spring mean temperatures, PICO and PIEN were separated farther by spring mean temperatures (Figure 3.5b). It showed that each species occupies a unique thermal niche related to their natural habitats. The  $T_{opt\_TL}$  also showed that PICO and PIEN from different sites had overlapping elevations but different  $T_{opt\_TL}$  ranges (Figures 3.5c) and fewer overlapping elevations after converting the elevation to spring mean temperatures (Figures 3.5d). It also showed that  $T_{opt\_TL}$  was more diverse than  $T_{opt\_GP}$  within each species (Figure 3.5).

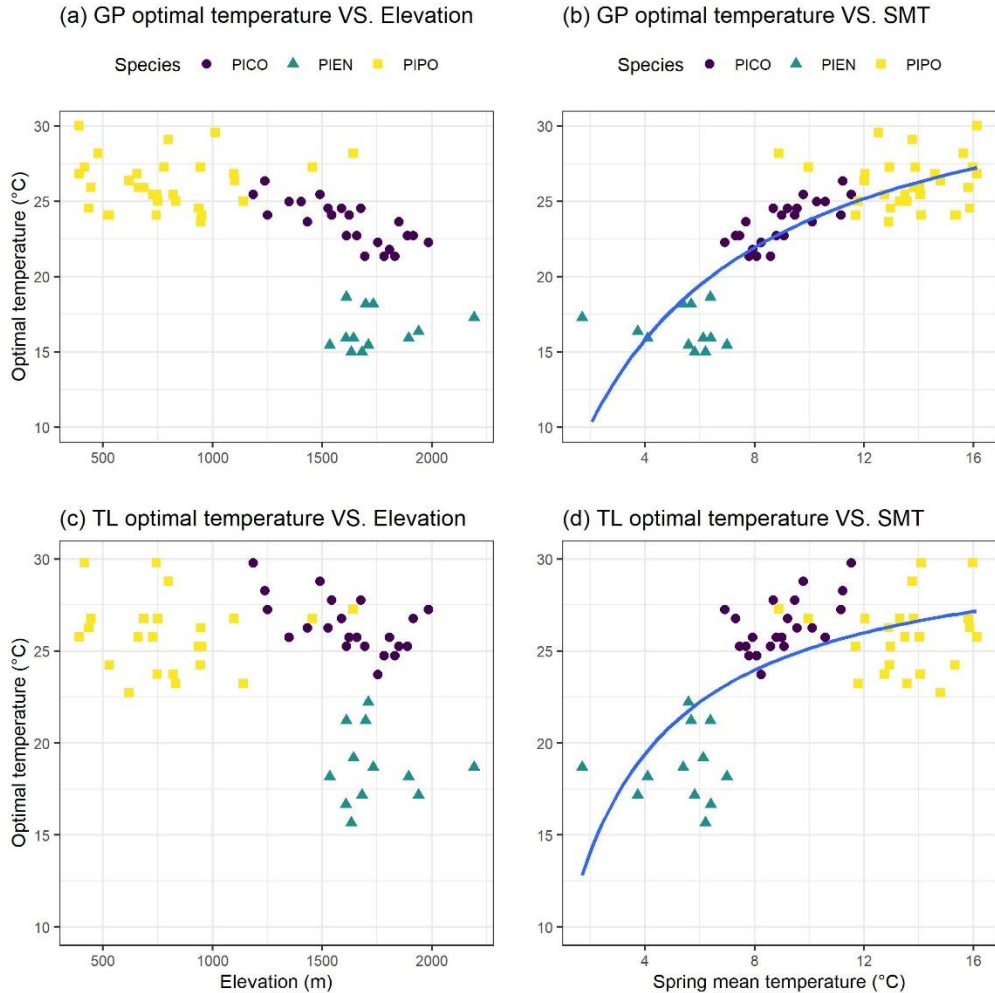


Figure 3.5 The germination percentages (GP) and tube length (TL) optimal temperatures of *Pinus contorta* (PICO), *Picea engelmannii* (PIEN), and *Pinus ponderosa* (PIPO) along the elevational gradient (a and c) and the spring mean temperature (SMT) gradient (b and d). The blue lines are Michaelis-Menten equations.

### 3.3.4 $T_{opt\_GP}$ and $T_{opt\_TL}$ are correlated.

The  $T_{opt\_TL}$  in each species was higher than  $T_{opt\_GP}$  (Tables 3.3 and S3.6), and these optimal temperatures had a positive relation (Figure 3.6). In other words, the populations with higher  $T_{opt\_GP}$  would also have higher  $T_{opt\_TL}$  (Figure 3.6). The  $T_{opt\_TL}$  was 2.3 to 2.9°C higher than the  $T_{opt\_GP}$  in PICO and PIEN, but not much difference in PIPO (Table 3.3). The  $t$ -tests between  $T_{opt\_GP}$  and  $T_{opt\_TL}$  in each species showed that  $T_{opt\_TL}$  was significantly higher than  $T_{opt\_GP}$  in PICO ( $p < 0.001$ ) and PIEN ( $p < 0.001$ ), and no difference between  $T_{opt\_GP}$  and  $T_{opt\_TL}$  in PIPO ( $p = 0.123$ ).

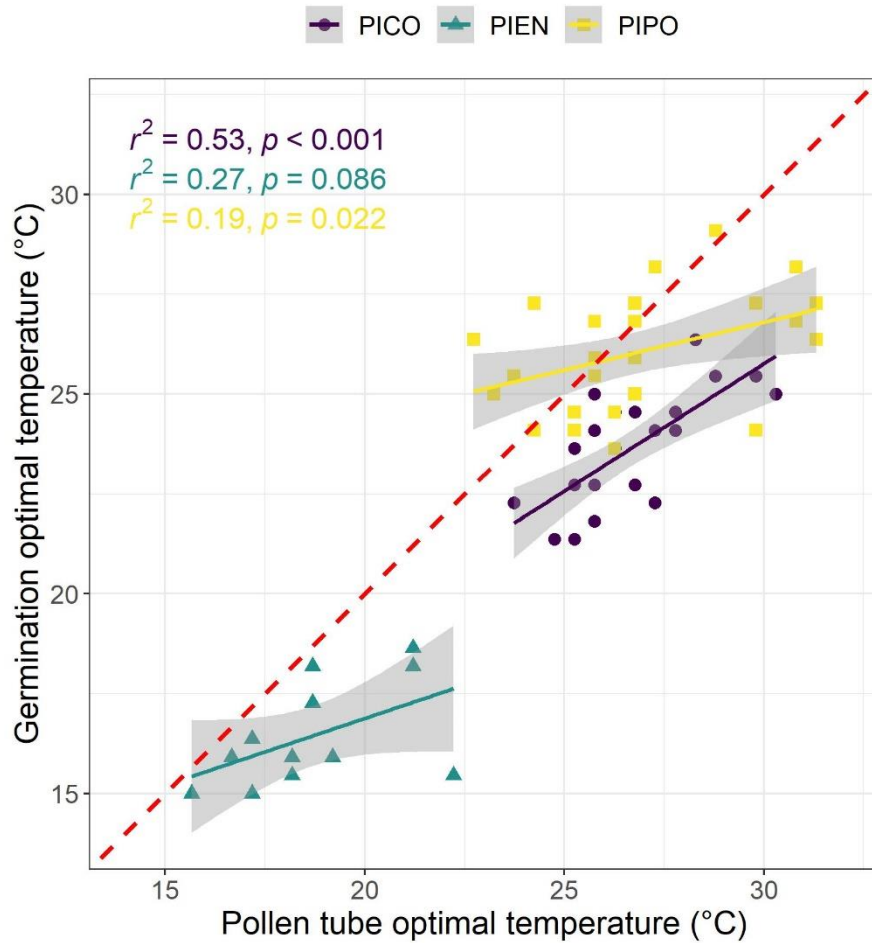


Figure 3.6 Simple linear regressions between the optimal temperatures of pollen germination percentage and tube lengths in *Pinus contorta* (PICO), *Picea engelmannii* (PIEN), and *Pinus ponderosa* (PIPO) across the elevational gradients. The red dash line is the 1:1 temperature ratio. The shaded areas are 95% confidence intervals.

### 3.4 Discussions

#### 3.4.1 Intraspecific variations of pollen GP and TL in each temperature along the elevational gradient.

Based on our  $H_1$ , we expect that at lower temperatures, the population from higher elevations (cooler sites) should have a higher GP, and those from lower elevations (warmer sites) should have a lower GP. That is, GP should have a positive slope along the elevational

gradient if the temperatures are lower than their optimal temperatures. On the contrary, it should have reversed trends (negative slope) along the elevational gradient at a temperature higher than their optima because the temperature range of pollen germination and tube growth reflects the population acclimation to the local environment (Hedhly et al., 2004, 2005b; Parantainen & Pulkkinen, 2002; Pasonen et al., 2000; Pham et al., 2015). In addition, when the temperature is near optimal, all populations should have similar GP, and there should not be much germination in pollen at extreme temperatures; hence, there should be no differences in GP along the elevational gradient (a slope close to zero) near optimal temperature and at extreme temperatures.  $T_{opt\_GP}$  of PICO is 23.6°C (Table 3.3). The trends along the elevational gradient follow our expectation that has a slope close to zero at temperature extremes (5, 35, and 40°C) and near-optimal (25°C), positive slopes at temperatures lower than optimal (10, 15, and 20°C), and negative slopes at temperatures higher than optimal (25 and 30°C; Table 3.2 and Figure S3.6). TL of PICO has a similar trend that slopes are close to zero at 5 and 10°C, positive slopes at 15 and 20°C, and a negative slope at 25°C, and slopes that are close to 0 at 30, 35, and 40°C (Table 3.2 and Figure S3.7). GP of PIEN and PIPO also show similar trends but different patterns due to their unique niche (Table 3.2, Figures S3.8 and S3.11). For example, the  $T_{opt\_GP}$  of PIEN is 16.4°C, but PIEN does not have a positive slope in the tested temperatures lower than  $T_{opt\_GP}$  (Table 3.2 and Figure S3.8), and PIPO has a narrower positive-slope temperature range (20°C) than PICO (10 to 20°C) (Table 3.2, Figures S3.10 and S3.6). There might be a narrow positive-slope temperature range for PIEN between 5 and 10°C that our experiment did not catch. It is also possible that PIEN is one of the species that occupy subalpine regions, and higher elevation means a harsher environment, resulting in lower pollen quality at higher elevations. Overall, our results in GP and TL of PICO and PIPO support our  $H_1$  that the population from higher elevations (cooler sites) will have higher germination percentages and longer tube lengths at lower temperatures and vice versa (Table 3.2 and Figures S3.6 to S3.11).

In addition, we can see that the GP has smaller variations near optimal temperatures and larger variations when the temperature moves toward upper or lower limits in each species (Figures 3.3a, 3.3c, and 3.3e). We do not find similar discussions regarding GP variations within

the same temperature and across a range of temperatures in the literature related to pollen, but it has the same results as our study in seed germination (Hsu, Stuke, Bakker, Kim, unpubl. res.). In the optimal temperature range, germination is relatively consistent among populations, as the conditions are generally favorable for growth and development. In suboptimal temperature ranges, there is greater variation in germination among populations, as the conditions are less favorable for growth. At temperature limits, such as the minimum or maximum temperature tolerances of species in our study, there is even more significant variation in germination among populations. This is because the conditions are more extreme, and fewer populations can tolerate them. It is also possible that there are many zero germination percentages at extreme temperatures, resulting in a higher coefficient of variation. The zeros at extreme temperatures may increase or decrease the coefficient of variations depending on how many zeros are and what values are not zeros. In our case, zeros increase CV because there are many zeros, and the values that are not zeros are small. We can not confirm whether these variations at different temperatures have ecological significance or are mathematical artifacts in our study. Conversely, TL has larger variations near optimal temperature and smaller variations when the temperature moves toward upper or lower limits in each species (Figures 3.3b, 3.3d, and 3.3f). Pollen grains have to compete for access to the ovules when there are more pollen grains than there are ovules. Only the fastest-growing pollen grains are assumed to achieve successful fertilization, which consequently, is a determinative factor controlling the paternity of the seeds (Pasonen et al., 1999). Hence, our results suggest that seeds will have higher genetic diversity if the pollen germinates at the suboptimal than if the pollen germinates at the optimal temperature. The reason is that the TL variation is smaller at suboptimal, and every pollen has almost equal chances to fertilize ovules. On the other hand, the pollen of the fast-tube-growing genotype will dominate the successful fertilization at the optimal temperature. When faced with unpredictable environmental conditions, fluctuation in pollen behavior due to genetic variability or phenotypic plasticity is a beneficial trait that allows plant species to fertilize successfully in a broader range of climates on the one hand, but it is disadvantageous that plants do not become more adapted to the local environment on the other hand.

With increasing temperatures in the future, depending on what temperature is right now during pollen shedding, GP and TL can either increase or decrease, and so can the genetic diversity of seeds. For example, if the current temperature during pollen shedding is lower than the optimal temperature, climate warming will likely increase GP and TL and decrease the genetic diversity of seeds. On the contrary, if the current temperature during pollen shedding is higher than the optimal temperature, climate warming will likely decrease GP and TL and increase the genetic diversity of seeds if TL is still long enough to fertilize ovules successfully. According to our temperature data at the pollen collection sites during pollen shedding (1.7 to 13.7°C; Table S3.1), they are much lower than  $T_{opt\_GP}$  and  $T_{opt\_TL}$  (Table 3.3), and rising temperatures will increase GP and TL and decrease the genetic diversity of seeds. However, another factor to consider is that the actual fertilization site is inside the female cone (in vivo). Because tree canopy absorbs and retains radiant energy, their temperatures are 15-20°C greater than the air temperature (Flores-Rentería et al., 2018). Therefore, the temperature at the actual fertilization site is much higher than the air temperature. Rising temperatures will likely decrease GP and TL and increase the genetic diversity of seeds if TL is still long enough to fertilize ovules successfully. Either way, the rising temperature will eventually affect pollen performance and change the future population and plant community composition (Rosbakh & Poschlod, 2016). Another consideration is our spring temperature is based on the calendar, whereas plants respond to the temperature. The flowering phenology will also change, but we do not know if the male and female cones will respond to temperatures in the same direction and rate. Overall, the inclusion of a pollen perspective on temperature variation improves our understanding of existing patterns of plant biogeography and range-shift responses to climate change. Furthermore, many species distribution models still suffer from an essential lack of temperature- and species-specific ecological data and a mechanistic understanding of how environmental factors shape plant ecophysiology and current species distributions (Mondoni et al., 2015; Parmesan & Hanley, 2015). We suggest that the temperature requirements of PG and TL in vitro could be integrated into species distribution models as they can estimate reproduction success quantitatively based on the germination percentage and tube length

under specific temperature conditions, as Rosbakh & Poschlod (2016) proposed, but need further study.

### 3.4.2 Inter- and intraspecific variations in $T_{opt\_GP}$ and $T_{opt\_TL}$ and their relations to pollen collection elevations or the spring mean temperatures.

Based on our  $H_2$ , we expect the  $T_{opt\_GP}$  and  $T_{opt\_TL}$  to follow the trends that the population from higher elevations (cooler sites) should have lower optimal temperatures for pollen germination and tube growth. The  $T_{opt\_GP}$  and  $T_{opt\_TL}$  in PICO across the elevational gradient show a significant descending trend ( $p < 0.001$  and  $p = 0.004$  in Figures 3.4a and 3.4b). In other words, the population from higher elevations has lower optimal temperature and vice versa, which suggests PICO populations have acclimated or adapted to their habitats (Hedhly et al., 2004, 2005b; Parantainen & Pulkkinen, 2002; Pasonen et al., 2000; Pham et al., 2015). However, no significant trends are found in GP and TL along the elevational gradient in PIEN ( $p = 0.553$  and  $p = 0.886$ ) and PIPO ( $p = 0.649$  and  $p = 0.419$ ) (Figures 3.4c to 3.4f). Our  $H_2$  is supported in PICO but not PIEN and PIPO. One explanation is that pollen collection sites in our study came from a relatively narrower geographic range that does not cover the whole range of the species. It is also possible that some other factors (e.g., microenvironment) affect the pollen germination and dilute the effect of elevation. Anderegg (2023) had similar opinions that several reasons may explain inconsistent and weak trait-climate relationships, including (1) incomplete sampling niche; (2) confounding factors that are difficult to disentangle geographically; (3) micro- and macro-climate variation decouples the environment that sampled individual actually experience from environmental predictor used in the analysis; and (4) nonadaptive trait variation.

PIEN occupies one of the highest and coldest forest environments in the western United States. Our results show that PIEN also has the lowest GP and TL optimal temperature, followed by PICO and PIPO (Table 3.3). The PIEN has a distinct elevation, and PICO and PIPO have overlapping ranges (Burns & Honkala, 1990), and the  $T_{opt\_GP}$  and  $T_{opt\_TL}$  of each species are similar to their bioclimatic ranges. (Figure 3.5). PICO and PIEN from different sites had

overlapping elevations but different  $T_{opt\_GP}$  and  $T_{opt\_TL}$  ranges (Figures 3.5a and 3.5c). After converting the elevations to actual spring mean temperatures, there is no overlapping between PICO and PIEN (Figures 3.5b and 3.5d). These results suggest that species have acclimated or adapted to the environment they occupy (Hedhly et al., 2004, 2005b; Parantainen & Pulkkinen, 2002; Pasonen et al., 2000). Pham et al. (2015) had similar opinions that species' origin can explain the temperature range for pollen germination. Our results show that each species occupies a unique thermal niche related to its natural habitats. Our  $H_2$  is supported.

A popular notion is that in the species or populations from colder regions, where the growing season is relatively short, pollen must either begin germinating at a lower temperature or grow pollen tubes faster than pollen from the warmer region (Varis et al., 2011). Our results support these explanations that PIEN has the lowest  $T_{opt\_GP}$  and  $T_{opt\_TL}$  (Table 3.3) and faster tube growth (approx. 180-260 $\mu$ m, peak values in Figure S3.4d) than PICO (approx. 100-160 $\mu$ m, peak values in Figure S3.4b) and PIPO (approx. 90-170 $\mu$ m, peak values in Figure S3.4f). A study of different species in the Bavarian Alps along an elevational gradient also found a strong positive relationship between temperature conditions at pollen collection sites and the minimum temperature for both pollen germination and pollen tube growth and a significant correlation between the maximum temperature of pollen tube growth and temperature of flowering month (Rosbakh & Poschlod, 2016).

### 3.4.3 Correlation between $T_{opt\_GP}$ and $T_{opt\_TL}$ .

The populations with higher  $T_{opt\_GP}$  also have higher  $T_{opt\_TL}$  (Figure 3.6). Kakani et al. (2002) found that minimum and maximum temperatures for pollen germination and pollen tube length were correlated, reflecting the overall adaptation of plants to extreme temperatures; however, the optimal temperatures were not correlated. The  $t$ -tests between  $T_{opt\_GP}$  and  $T_{opt\_TL}$  in each species show that  $T_{opt\_TL}$  is significantly higher than  $T_{opt\_GP}$  in PICO ( $p < 0.001$ ) and PIEN ( $p < 0.001$ ), but no differences between  $T_{opt\_GP}$  and  $T_{opt\_TL}$  in PIPO ( $p = 0.123$ ). Our  $H_3$  is mostly supported, except the  $T_{opt\_GP}$  and  $T_{opt\_TL}$  have no difference in PIPO. Some studies also found that  $T_{opt\_TL}$  is higher than  $T_{opt\_GP}$  (Rosbakh & Poschlod, 2016; Steinacher & Wagner, 2012). Pollen

of *Pinus* may germinate soon after pollination, and pollen tubes penetrate the nucellus. After that, the seed cone and pollen tubes become dormant by midsummer and resume growth the following spring (Fernando et al., 2005). Therefore, our observations might be related to the fact that pollen tubes grow after pollen germination, resulting in pollen tube growth acclimating or adapting to a higher temperature in the later season. Temperature strongly affects the tube growth rate in the conifer species in our study. Many studies have reported that pollen tubes grow faster with rising temperatures for various tree species (Hedhly et al., 2005a, 2005b; Pasonen et al., 2000) because pollen tubes are the fastest-growing plant cells known, and their growth is highly dependent on energy production and biosynthetic capacity (Gass et al., 2005). These metabolic processes are related to proteins whose rates are highly influenced by temperature. That is, pollen tube grows fast with rising temperatures within the optimal temperature range, and growth rates decrease at low and high temperatures. However, the reasons for decreasing rates at low and high temperatures are different. At cold but non-freezing temperatures, slow tube growth primarily results from the delay of metabolic process, and the tubes promptly resume growth and fertilization occurs as temperatures increase; conversely, high temperatures lead to irreversible functional disruptions, resulting in abnormal tube shapes or polarity issues (Steinacher & Wagner, 2012).

### 3.5 Conclusions

Pollen germination and tube growth are highly sensitive to climatic conditions and may vary among species and populations. Our results illustrate the temperature dependence of pollen germination and tube growth across a wide range of temperatures of three conifer species along an elevational gradient in the eastern Cascades of Washington. They show that pollen from different species and different elevations within the same species had different germination percentages, tube lengths, and optimal temperatures correlated with their ecological niche. In addition, the population with a higher optimal temperature for germination will also have a higher optimal temperature for pollen tube growth, and the tube growth optimal temperature is higher than the optimal temperature for germination. We also find that

the variations of pollen germination are larger when the temperature approaches the upper and lower biological limits of germination. Conversely, the variations of tube growth are larger when the temperature is optimal. Our understanding of pollination in conifers has advanced rapidly in the past two decades, but it still falls behind our knowledge of this process in flowering plants. To be able to better explain how temperature responses of pollen performance vary among conifer species and within species under climate change, we suggest future studies in the pollination mechanism of conifers, the influence of pollen-pollen interaction on fertilization abilities, other environmental factors (e.g., relative humidity) related to pollination mechanism in different conifer genera, and the plasticity of response on the individual level. Determining the temperature dependence of conifer pollen germination and pollen tube growth from different conifer species and populations is essential to evaluating species' response to temperature for predicting and mitigating the impacts of climate change on plant populations and ecosystems. Nevertheless, temperature-induced reproductive disorder and failure, e.g., mismatches with seasonality and inability of pollen to germinate, etc., are rarely considered in species distribution models because this species-specific information is hard to obtain. Our study on the temperature requirements of pollen germination and tube growth in vitro could be integrated into species distribution models as they can estimate reproduction success quantitatively under specific temperature conditions. We anticipate these results will provide the information needed to improve current conifer species distribution models and help researchers, policymakers, and stakeholders develop climate adaptation strategies.

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Supplementary materials

Table S3.1. Climatic conditions of nature habitats (Burns and Honkala, 1990) and pollen collection sites (self-collected) of each species. MAP: mean annual precipitation; MST: mean spring (April to June) temperature.

Species	<i>Picea engelmannii</i>	<i>Pinus contorta</i>	<i>Pinus ponderosa</i>
<b>Nature habitats</b>			
Temperature range	1. MAT: -1 to 2°C	1. Min T: -57 to 7°C	1. MAT: 5 to 10°C
	2. Mean Jan T: -12 to -7°C	2. Max T: 27 to 38°C	2. Mean Jul-Aug T: 17 to 21°C
	3. Mean Jul T: 4 to 16°C		
MAP range	610 to 4060 mm	250 to 500 mm	280 to 1750 mm
<b>Pollen collection sites</b>			
MST range	1.7 to 7.4°C	3.4 to 6.1°C	7.1 to 13.7°C

Table S3.2. Formula for pollen culture medium (Brewbaker and Kwack, 1963; Varis et al., 2011)

Chemicals	Concentration
Sucrose	10% (100 g/l)
Boric acid	100 mg/l
Calcium nitrate	300 mg/l
Magnesium sulfate	200 mg/l
Potassium nitrate	100 mg/l
Thiamine	50 mg/l
Riboflavin	25 mg/l
Ascorbic acid	50 mg/l

Table S3.3. The total number of samples in each species.

Species	Temperatures	Elevations	Rounds	Total samples	Subsamples	Totals
PICO		23		552		1104
PIEN	8	12	3	288	2	576
PIPO		30		720		1440

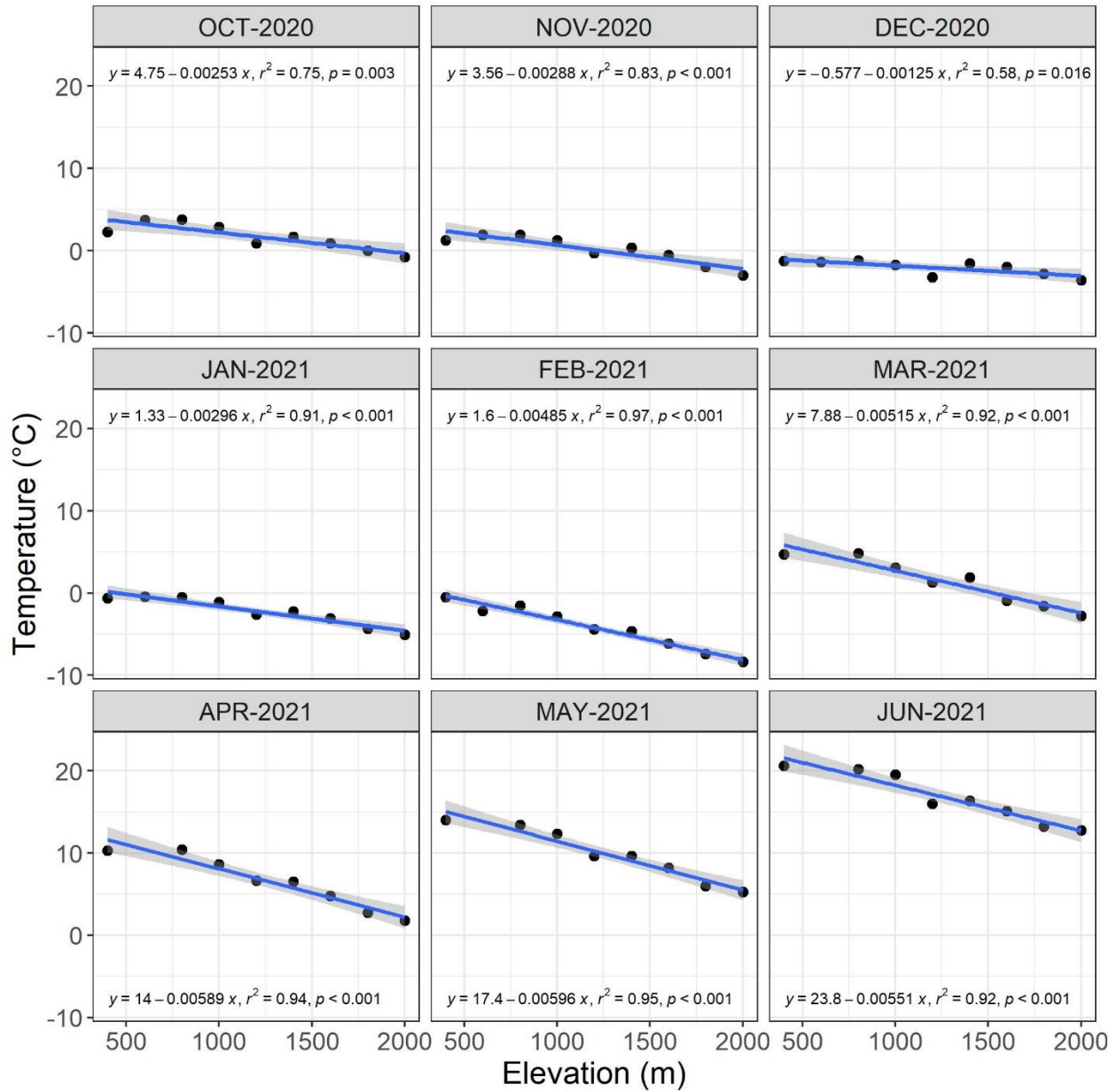


Figure S3.1. The monthly mean temperature along the elevation and lapse rate in Tyee Mountain. The shaded areas are 95% of confidence intervals.

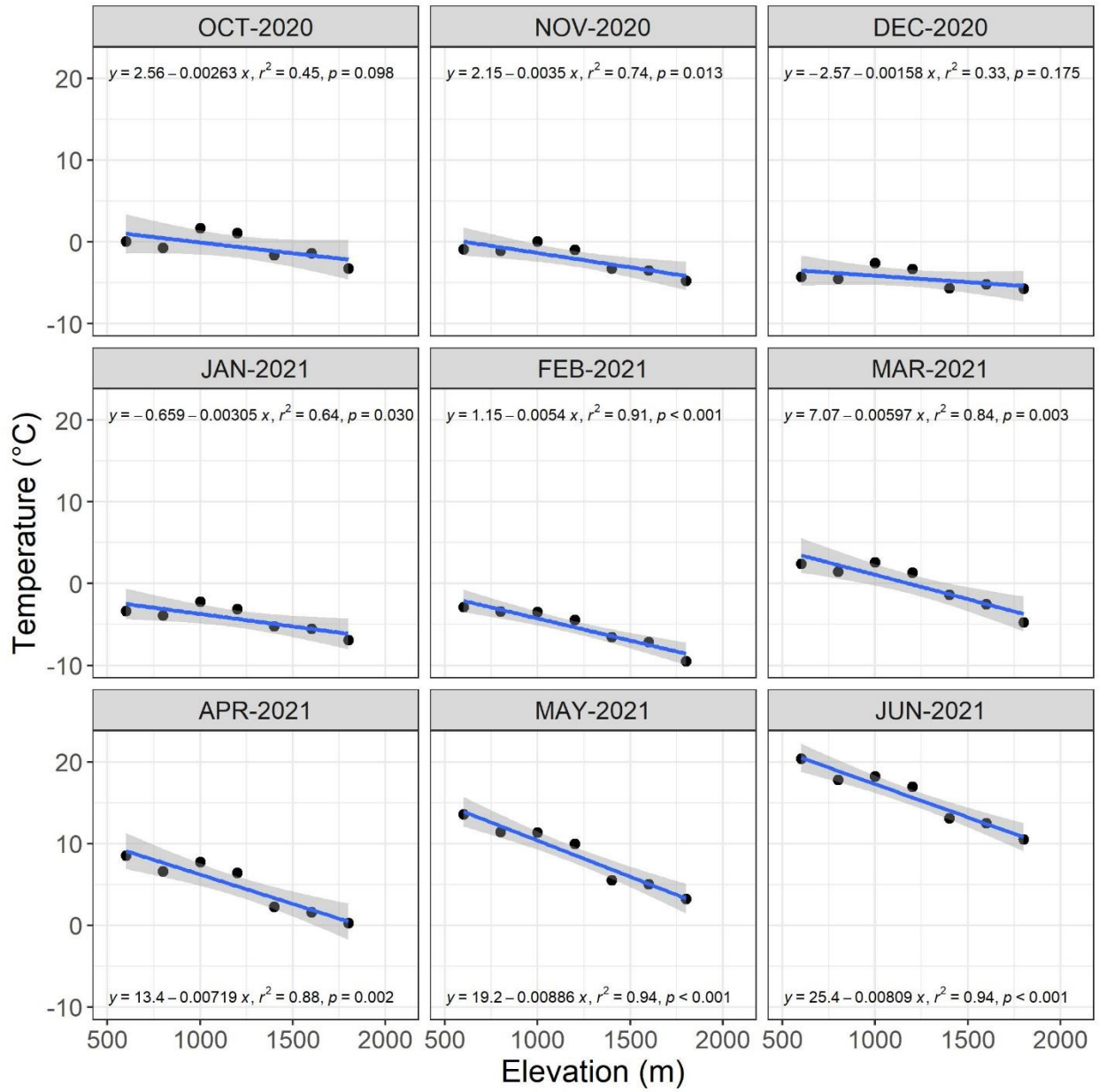


Figure S3.2. The monthly mean temperature along the elevation and lapse rate in Slate peak. The shaded areas are 95% of confidence intervals.

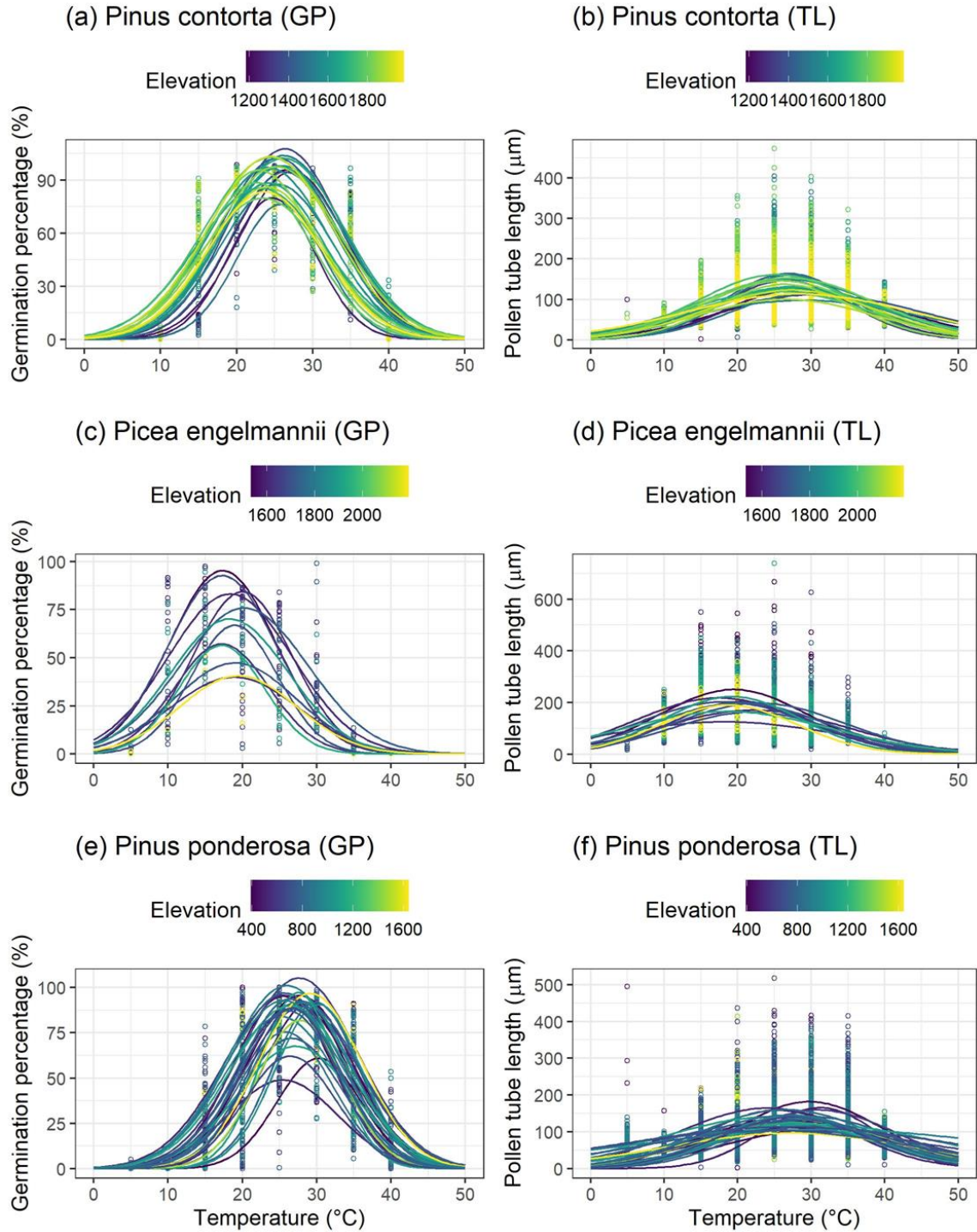


Figure S3.3. Gaussian temperature response curves of pollen germination percentages (GP) and tube lengths (TL) in *Pinus contorta* (PICO; a and b), *Picea engelmannii* (PIEN; c and d), and *Pinus ponderosa* (PIPO; e and f) from different elevations (PICO: 23, PIEN: 12, PIPO: 30 elevations).

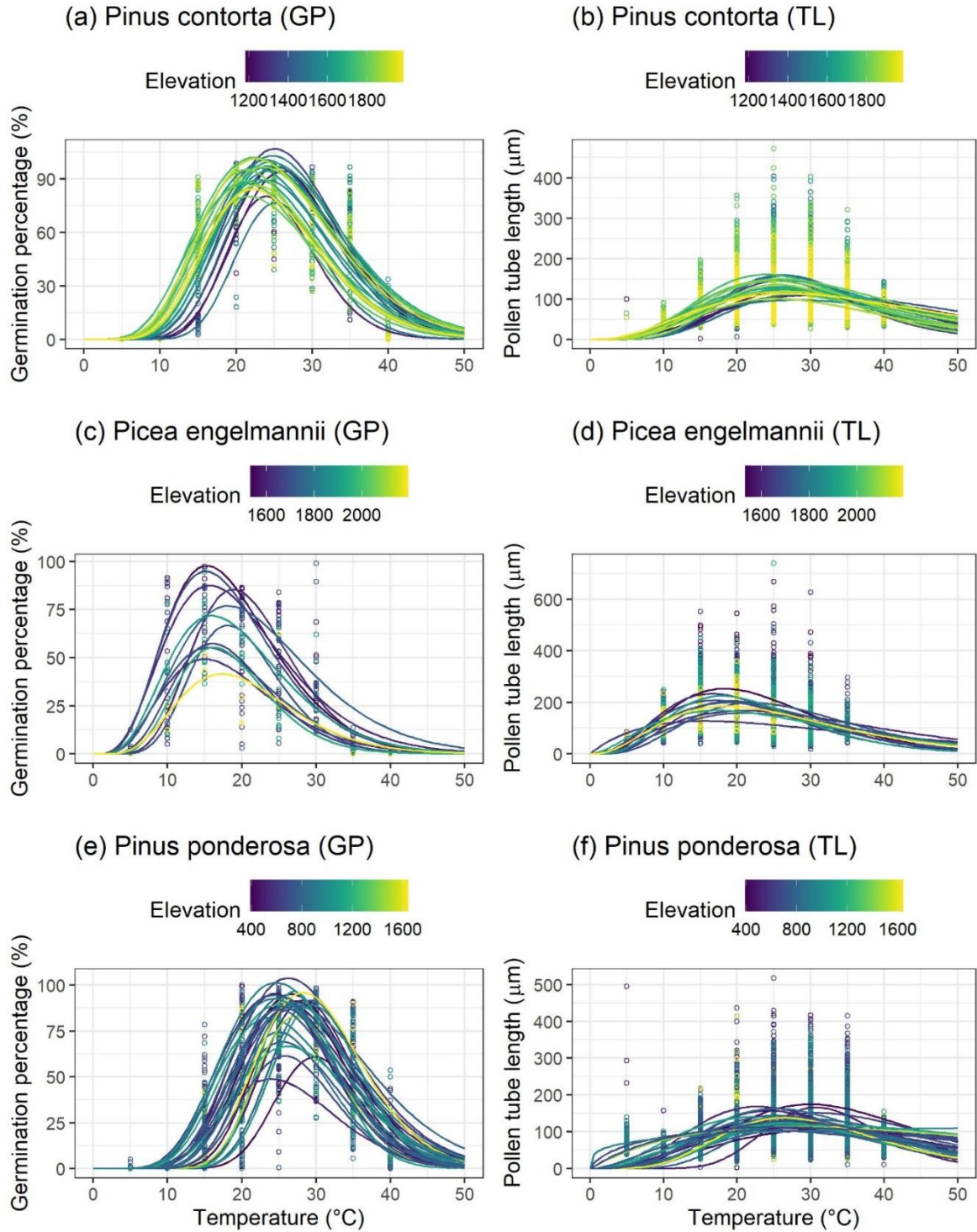


Figure S3.4. Gamma temperature response curves of pollen germination percentages (GP) and tube lengths (TL) in *Pinus contorta* (PICO; a and b), *Picea engelmannii* (PIEN; c and d), and *Pinus ponderosa* (PIPO; e and f) from different elevations (PICO: 23, PIEN: 12, PIPO: 30 elevations).

Table S3.4. AIC comparisons ( $\Delta$ AIC) between fitted Gaussian and Gamma curves of pollen germination percentage and different temperatures in each elevation. Note: AIC is only compared for the same species and elevation.

PICO			PIEN			PIPO		
Elevation (m)	$\Delta$ AIC		Elevation (m)	$\Delta$ AIC		Elevation (m)	$\Delta$ AIC	
	Gaussian	Gamma		Gaussian	Gamma		Gaussian	Gamma
1184	3.3	0	1534	11.7	0	391	0.5	0
1238	0	4.5	1608	10.4	0	392	0	1.3
1250	3.3	0	1610	7.1	0	416	0	12.2
1349	0	5.1	1633	2.4	0	435	3.4	0
1404	7.4	0	1643	7.7	0	445	1.2	0
1432	0	2.2	1682	15.5	0	477	0	3.9
1489	0	0.5	1698	0.0	0	521	0	1.0
1527	0	2.8	1710	3.5	0	529	9.6	0
1542	0	5.4	1733	3.2	0	620	7.0	0
1587	0	5.5	1895	11.6	0	655	0	5.7
1610	8.2	0	1940	0	0.9	661	0	3.8
1623	0	3.0	2192	0.5	0	687	0	3.3
1658	14.9	0				727	0	0.6
1676	0	4.2				743	8.1	0
1694	9.8	0				747	0	1.1
1753	5.8	0				750	11.4	0
1781	14.6	0				778	0	4.0
1807	10.2	0				798	0	3.8
1831	5.7	0				819	0	4.8
1850	1.1	0				832	0	8.1
1888	11.8	0				936	0.3	0
1915	4.1	0				944	2.4	0
1984	10.1	0				947	3.6	0
						950	0	0.8
						1012	0	2.8
						1097	0	0.1
						1101	0.8	0
						1140	0	1.2
						1455	0.2	0
						1642	10.5	0

Table S3.5. AIC comparisons ( $\Delta$ AIC) between fitted Gaussian and Gamma curves of pollen tube length and different temperatures in each elevation. Note: AIC is only compared for the same species and elevation.

PICO			PIEN			PIPO		
Elevation (m)	$\Delta$ AIC		Elevation (m)	$\Delta$ AIC		Elevation (m)	$\Delta$ AIC	
	Gaussian	Gamma		Gaussian	Gamma		Gaussian	Gamma
1184	0	0	1534	0.1	0	391	14.2	0
1238	0	25.9	1608	49.1	0	392	22.1	0
1250	0	0.2	1610	0	5.3	416	31.8	0
1349	0	5.6	1633	0	1.0	435	14.2	0
1404	2.6	0	1643	10.9	0	445	14.0	0
1432	0	22.2	1682	17.9	0	477	54.0	0
1489	0	10.6	1698	0	8.5	521	-	-
1527	0	16.1	1710	1.1	0	529	4.4	0
1542	9.9	0	1733	3.6	0	620	0	17.1
1587	0	19.5	1895	14.0	0	655	51.3	0
1610	3.4	0	1940	8.1	0	661	3.4	0
1623	3.0	0	2192	0	5.6	687	76.9	0
1658	0	6.1				727	3.5	0
1676	0	23.2				743	7.2	0
1694	20.7	0				747	0	2.9
1753	11.4	0				750	23.9	0
1781	0.2	0				778	6.6	0
1807	0	0.3				798	40.7	0
1831	4.1	0				819	11.6	0
1850	0	9.0				832	16.3	0
1888	5.9	0				936	6.2	0
1915	0	21.8				944	0	0.5
1984	3.7	0				947	8.3	0
						950	95.0	0
						1012	3.3	0
						1097	6.2	0
						1101	4.9	0
						1140	22.8	0
						1455	0	1.8
						1642	0	5.6

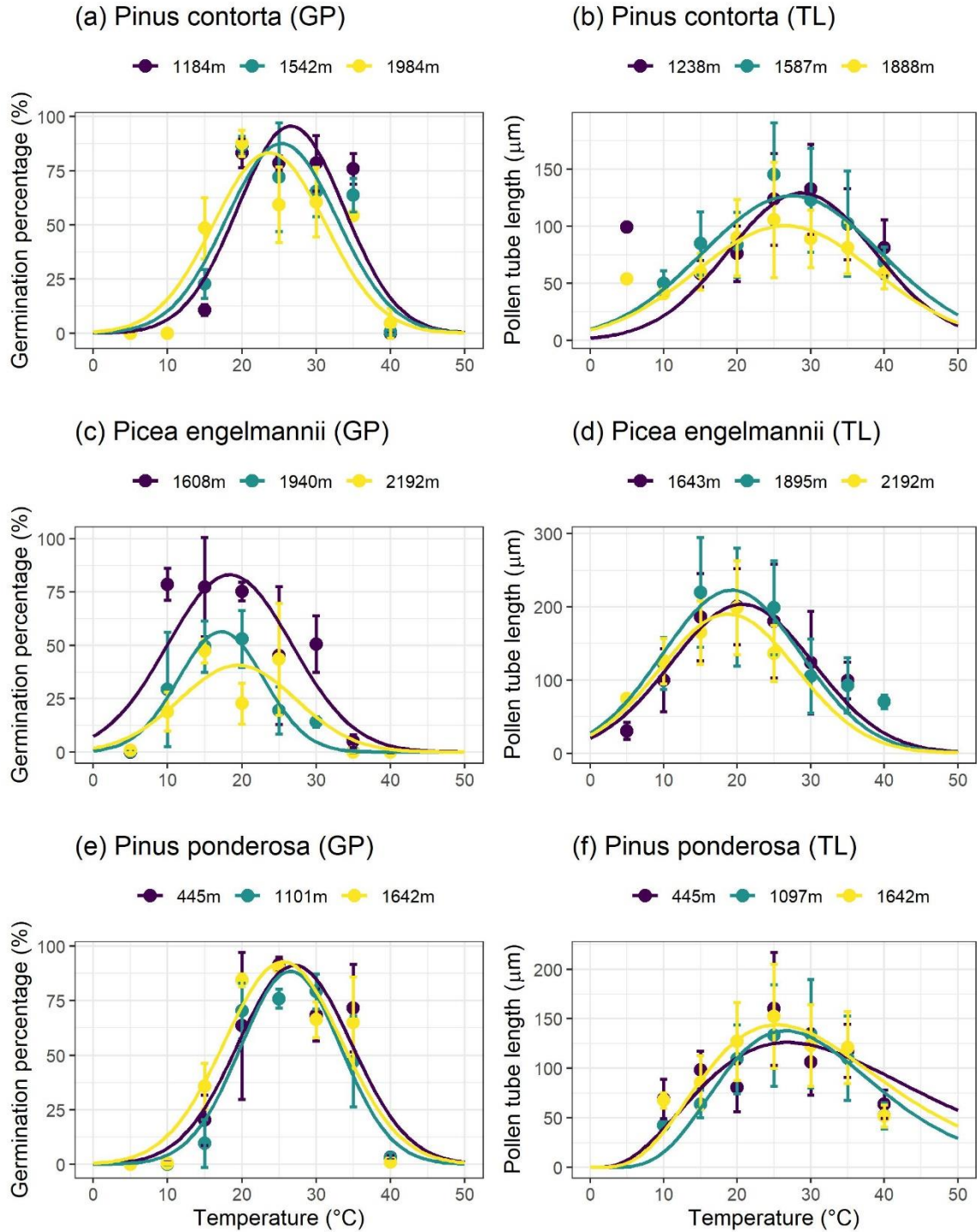


Figure S3.5. Gaussian temperature response curves of pollen germination percentages (GP) and tube lengths (TL) in *Pinus contorta* (PICO; a and b), *Picea engelmannii* (PIEN; c and d), and *Pinus ponderosa* (PIPO; e and f) from three elevations (low, medium, and high) of each species that is close to the regressions lines in Figure 3.4.

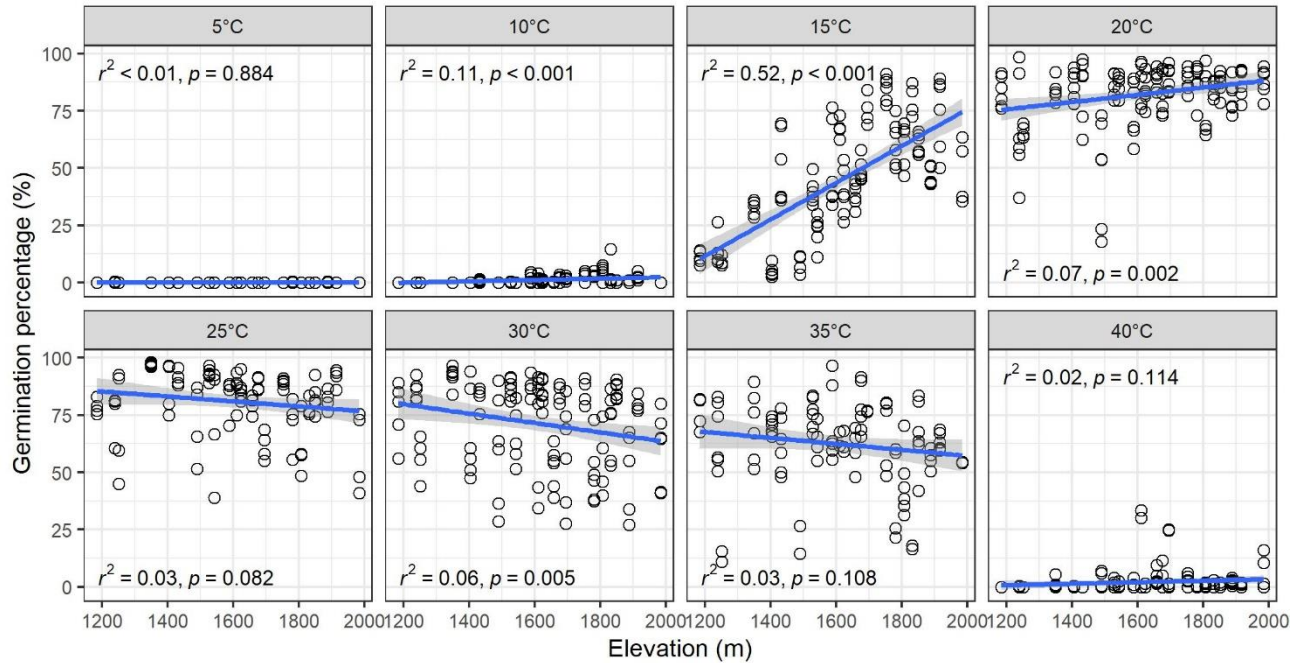


Figure S3.6. Germination percentages of *Pinus contorta* (PICO) at different temperatures along the elevational gradient. The shaded areas are 95% confidence intervals.

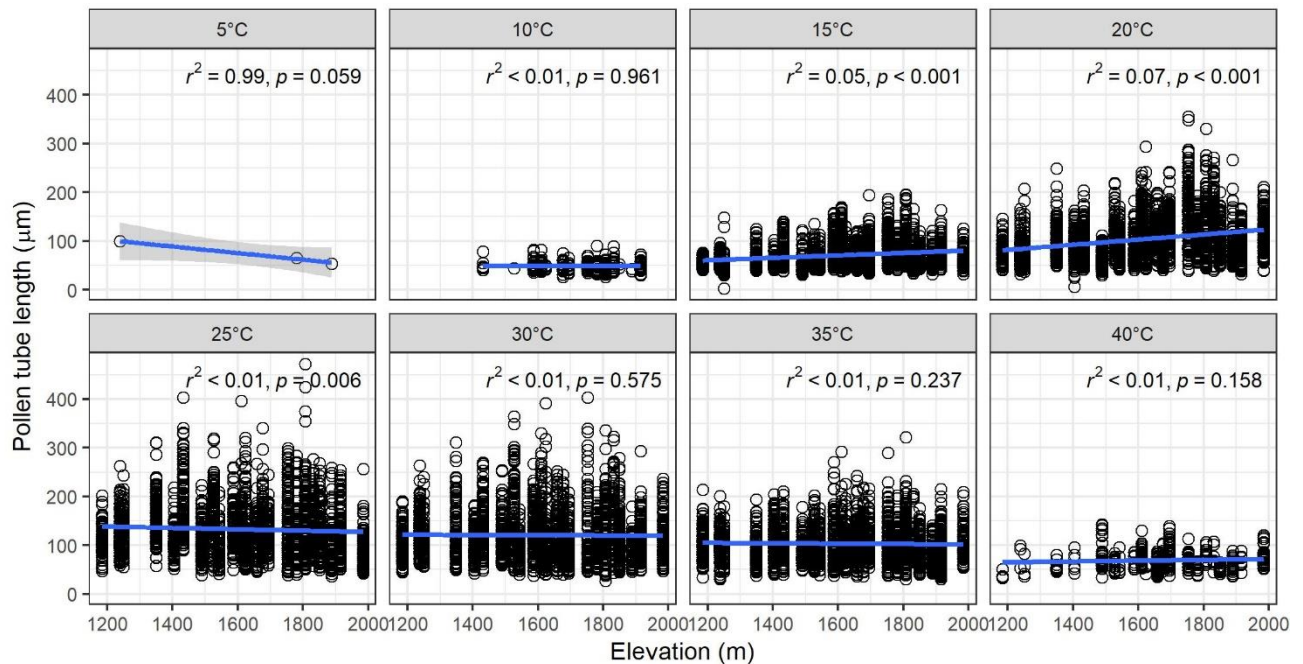


Figure S3.7. Pollen tube lengths of *Pinus contorta* (PICO) at different temperatures along the elevational gradient. The shaded areas are 95% confidence intervals.

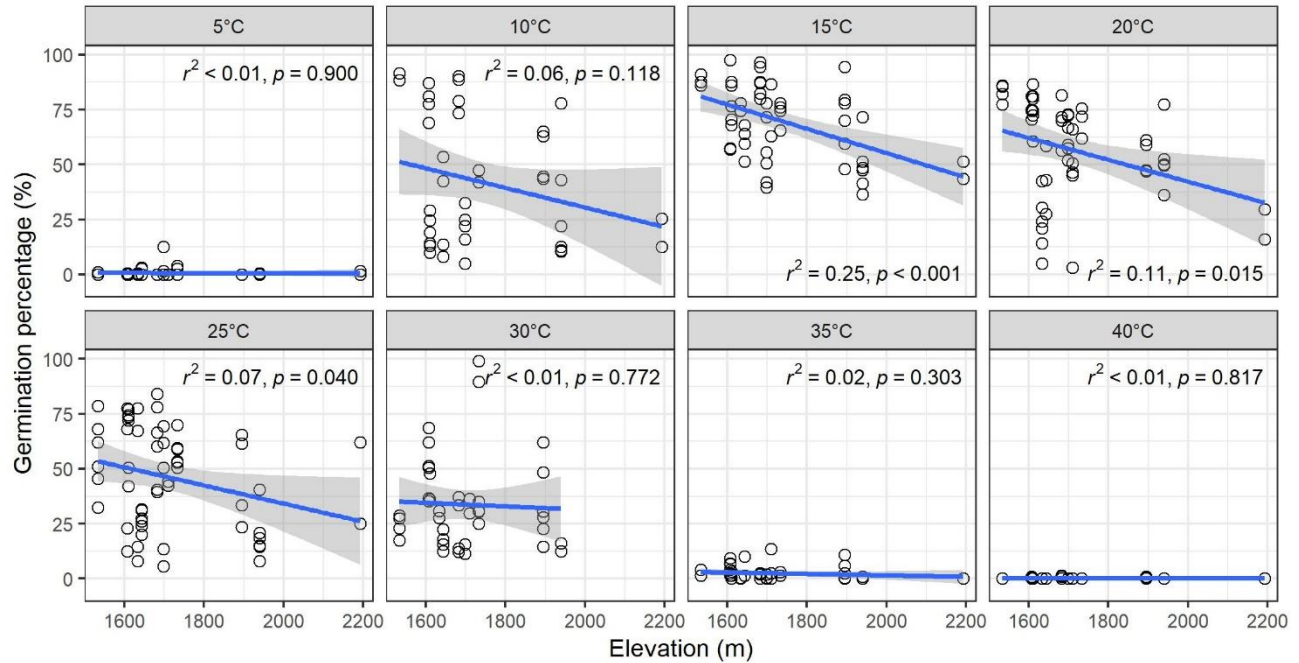


Figure S3.8. Germination percentages of *Picea engelmannii* (PIEN) at different temperatures along the elevational gradient. The shaded areas are 95% confidence intervals.

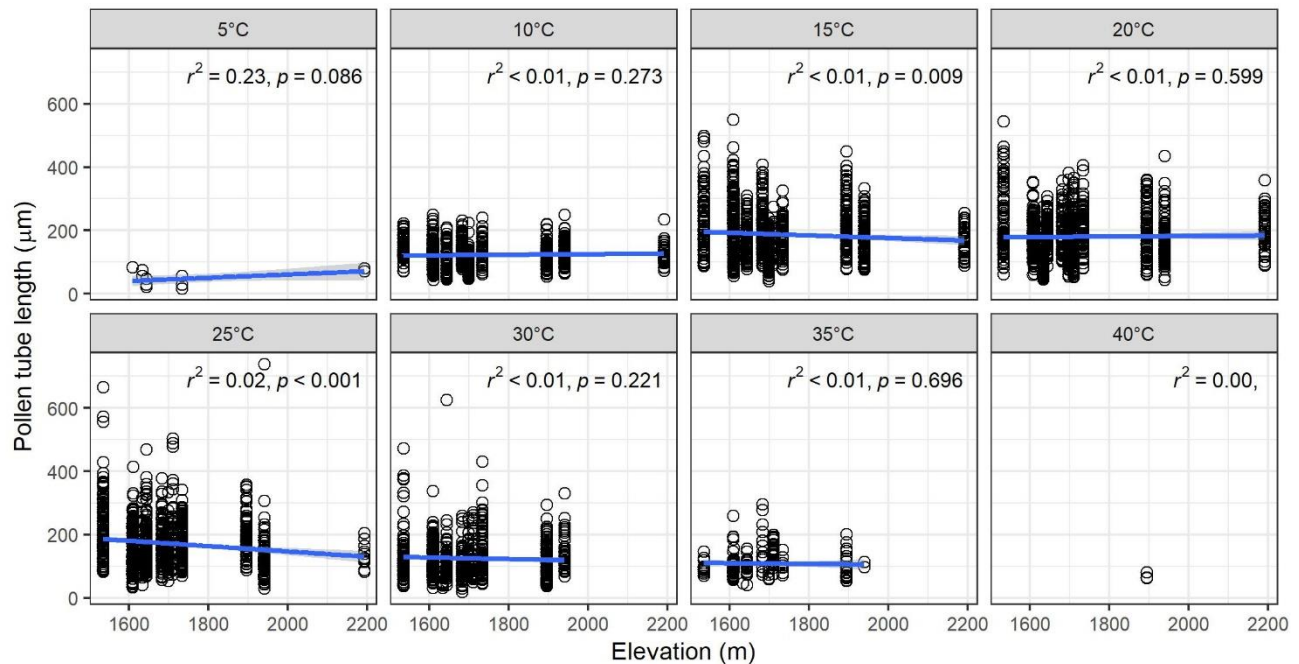


Figure S3.9. Pollen tube lengths of *Picea engelmannii* (PIEN) at different temperatures along the elevational gradient. The shaded areas are 95% confidence intervals.

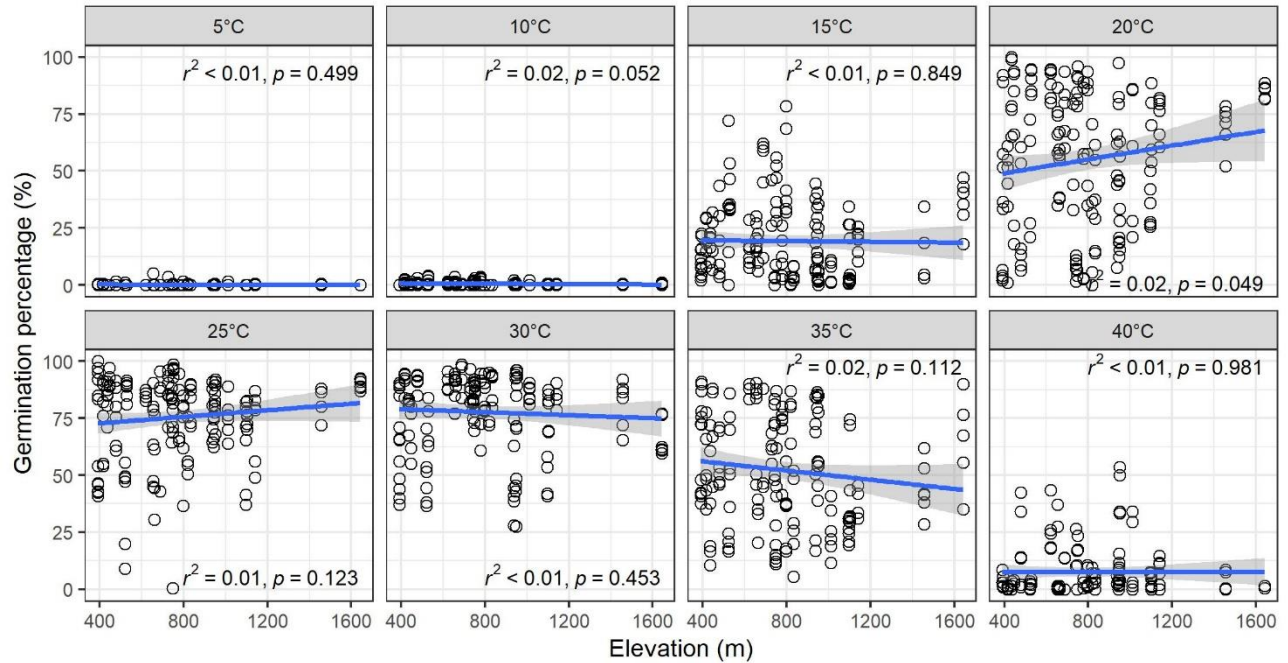


Figure S3.10. Germination percentages of *Pinus ponderosa* (PIPO) at different temperatures along the elevational gradient. The shaded areas are 95% confidence intervals.

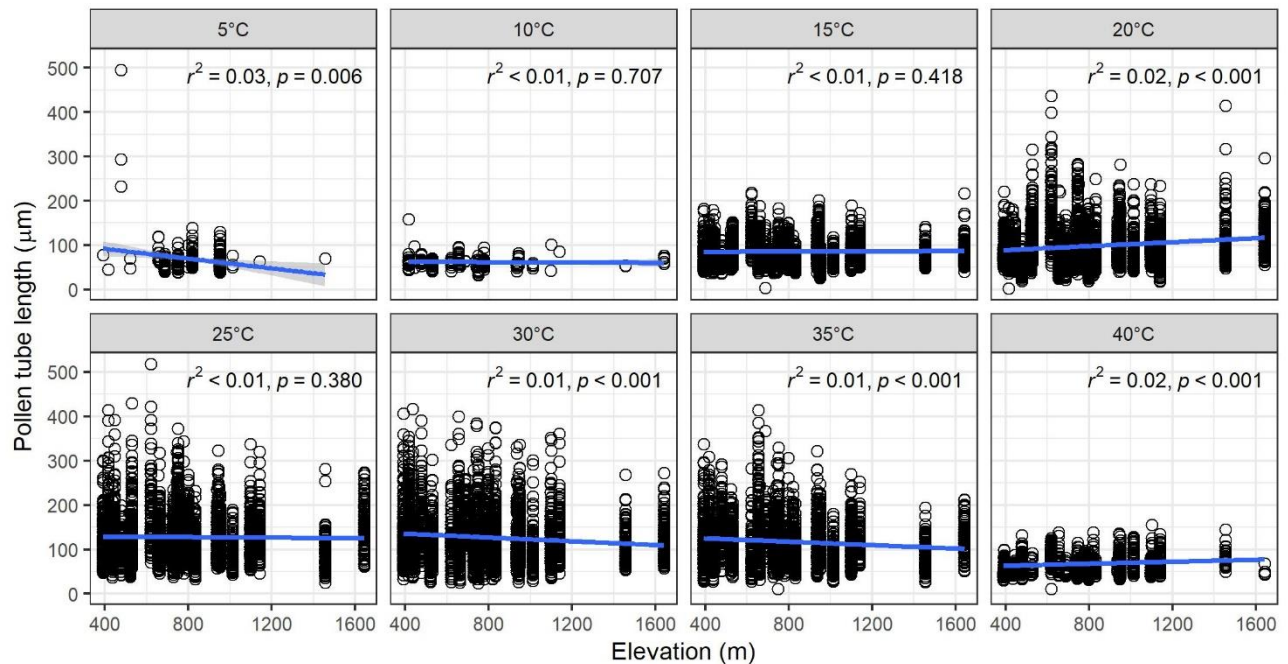


Figure S3.11. Pollen tube lengths of *Pinus ponderosa* (PIPO) at different temperatures along the elevational gradient. The shaded areas are 95% confidence intervals.

Table S3.6. The Gamma function derived optimal temperatures for pollen germination percentages ( $T_{opt\_GP}$ ) and tube lengths ( $T_{opt\_TL}$ ) of *Pinus contorta* (PICO), *Picea engelmannii* (PIEN), and *Pinus ponderosa* (PIPO) at each elevation.

PICO			PIEN			PIPO		
Elevation (m)	$T_{opt\_GP}$ (°C)	$T_{opt\_TL}$ (°C)	Elevation (m)	$T_{opt\_GP}$ (°C)	$T_{opt\_TL}$ (°C)	Elevation (m)	$T_{opt\_GP}$ (°C)	$T_{opt\_TL}$ (°C)
1184	25.5	29.8	1534	15.5	18.2	391	30.0	32.3
1238	26.4	28.3	1608	15.9	16.7	392	26.8	25.8
1250	24.1	27.3	1610	18.6	21.2	416	27.3	29.8
1349	25.0	25.8	1633	15.0	15.7	435	24.5	26.3
1404	25.0	30.3	1643	15.9	19.2	445	25.9	26.8
1432	23.6	26.3	1682	15.0	17.2	477	28.2	30.8
1489	25.5	28.8	1698	18.2	21.2	521	24.1	-
1527	24.5	26.3	1710	15.5	22.2	529	24.1	24.2
1542	24.1	27.8	1733	18.2	18.7	620	26.4	22.7
1587	24.5	26.8	1895	15.9	18.2	655	26.8	30.8
1610	22.7	25.3	1940	16.4	17.2	661	25.9	25.8
1623	24.1	25.8	2192	17.3	18.7	687	25.9	26.8
1658	22.7	25.8				727	25.5	25.8
1676	24.5	27.8				743	24.1	29.8
1694	21.4	25.3				747	25.5	23.7
1753	22.3	23.7				750	25.0	26.8
1781	21.4	24.7				778	27.3	31.3
1807	21.8	25.8				798	29.1	28.8
1831	21.4	24.7				819	25.5	23.7
1850	23.6	25.3				832	25.0	23.2
1888	22.7	25.3				936	24.5	25.3
1915	22.7	26.8				944	27.3	24.2
1984	22.3	27.3				947	23.6	26.3
						950	24.1	25.3
						1012	29.5	-
						1097	26.8	26.8
						1101	26.4	31.3
						1140	25.0	23.2
						1455	27.3	26.8
						1642	28.2	27.3

## Chapter 4

### Temperature dependence of seed germination of four conifers in the Western United States

**Authors:**

Hsin-Wu Hsu<sup>1</sup>,

ORCID: 0000-0002-6347-4540

E-mail address: [hwhsu@uw.edu](mailto:hwhsu@uw.edu)

Miro Stuke<sup>1</sup>,

ORCID: 0009-0006-7019-2775

E-mail address: [stuke@uw.edu](mailto:stuke@uw.edu)

Jonathan D. Bakker<sup>1</sup>,

ORCID: 0000-0002-8973-2771

E-mail address: [jbakker@uw.edu](mailto:jbakker@uw.edu)

Soo-Hyung Kim<sup>1\*</sup>,

ORCID: 0000-0003-3879-4080

E-mail address: [soohkim@uw.edu](mailto:soohkim@uw.edu),

<sup>1</sup>School of Environmental and Forest Sciences, University of Washington, Seattle, WA 98195-4115, USA

\*Corresponding author

## Abstract

Seed germination is a vital process in the plant life cycle. It directly influences seedling establishment and subsequently determines population persistence and species distribution. Germination is highly sensitive to climatic conditions, and germination traits may vary among species and populations within the same species. Our study aims to understand intraspecific and interspecific variations in the temperature dependence of seed germination and how these variations are related to the environments at various seed collection locations. We focused on four conifer species (*Pinus contorta*, *Picea engelmannii*, *Pinus ponderosa*, and *Pseudotsuga menziesii*) with seeds collected at 20 sites that spanned the elevation range from 150 to 1705m in the Western United States. We conducted seed germination tests at different temperatures from 5 to 40°C in 5°C intervals and tracked germination for 30 days. We used the log-logistic time-to-event model and Gaussian function to fit germination data and their temperature dependence. The climate variables at the seed-collecting sites were calculated as averages from 1971 to 2000, covering most seed-collection years. They were characterized using Principal Component Analysis (PCA). PCA reduced climate variables into several composite variables or principal components (PCs) that explained the most climate variations in seed collection sites (PCA scores). The estimated optimal temperature ( $T_o$ ), shape coefficient ( $b$ ), and maximum value at optimal temperature ( $GP_{max}$ ) of germination percentages and median germination rates ( $GR_{50}$ ) were then fit to linear models to determine whether they varied with species identity and the climate-derived PCA scores in the first two PCs. Results showed that seed germination traits from different species exhibit climate gradients that are correlated with their ecological niche. However, seeds from different elevations within the same species had variable germination traits irrespective of environmental gradients. We also found that the variations of germination were more significant when the temperature approached upper and lower limits. We anticipate that this understanding can predict population dynamics and range shifts under climate warming and help adjust forest management practices, seed transfer, and assisted migration.

**Keywords:** Conifer seeds, Seed germination, Temperature dependence, Intraspecific variation, Climate change

## 4.1 Introduction

Seed germination is an essential process in the plant life cycle. It affects seedling survival and establishment and subsequently determines population persistence, community structure, and species distribution (Bykova et al., 2012; Cochrane et al., 2015a). Seed germination traits have been overlooked compared to frequently used morphological traits, e.g., seed mass, shape, size, etc., and should be incorporated into vegetation science to include functions that cannot be demonstrated solely by adult plant traits (Jiménez-Alfaro et al., 2016). Temperature is one of the most critical environmental factors that regulate seed germination speed, uniformity, and percentage. It affects the rate of chemical reactions and the enzyme activity that controls various seed metabolic processes. Seed germination can be affected by fluctuations in temperature, such as diurnal temperature or rapid seasonal temperature changes, and is consequently highly sensitive to changing climate (Fernández-Pascual et al., 2019, 2021; Yuan et al., 2023). In addition, optimal germination temperatures are determined by the conditions the seeds have adapted to in their natural environment (Chamorro et al., 2018). Therefore, the specific temperature requirements for germination vary depending on the plant species (Baskin & Baskin, 2014). Warming due to climate change may promote faster seed germination for species at high elevations if temperatures become optimal, inhibit germination in areas where temperatures surpass the optimum, or make it difficult for seeds to survive for species at low elevations where high temperatures may become extreme. Seed quality and germination capacity can vary considerably between individuals from different provenances or ecotypes within a single species (Chamorro et al., 2017; Cochrane et al., 2015a). This variation is also key to mitigating the species' vulnerability to changing climate and providing species adaptation and conservation opportunities (Chamorro et al., 2017; Cochrane et al., 2015a; Marcora et al., 2008; Messier et al., 2010; Sides et al., 2014; Wu et al., 2018). Therefore, determining the temperature dependence of seed germination from different provenances or seed sources is essential to evaluate species' response to temperature for predicting and mitigating the impacts of climate change on plant populations and ecosystems. However, intraspecific variation in the response of seed germination traits to environmental change is rarely studied despite its critical role in plant life history (Cochrane et al., 2015a).

The variability in seed germination is influenced by two crucial factors: genetics (G) and the environment (E), as experienced by the mother plant during seed maturation (Baskin & Baskin, 2014). Each seed contains a unique combination of genetic material that may result in variations in the plant's characteristics, e.g., size, shape, color, resistance to pests and diseases, etc., and potentially affect seed germination patterns. In addition, genetics interact with environmental conditions (G x E interactions) in shaping the germination process (Baskin & Baskin, 2014). Thus, determining the relative importance of genetics and environmental factors in the variation of seed germination for a specific species often demands extensive research and is challenging to tease apart. Seeds collected from various sites also exhibit differences in germination characteristics, and the germination responses vary based on physical, environmental, and geographic features where the seeds mature (Baskin & Baskin, 2014; Liu & El-Kassaby, 2015; Stoehr et al., 1998). Previous studies have shown considerable in seed germination percentage, mean germination time, or germination niche with respect to temperature within one single species (intraspecific variation) (Cochrane et al., 2014; Cochrane et al., 2015b) and between species (interspecific variation) (Gao et al., 2021; Skordilis & Thanos, 1995). The variabilities within and between species were correlated with elevation (Haasis & Thrupp, 1931; Roche, 1969), mean annual temperature (Cochrane et al., 2014), mean annual temperature and precipitation, and mean temperature and precipitation of the driest season (Gao et al., 2021).

Time-to-event methods refer to modeling the time until an event of interest occurs. In seed germination tests, researchers periodically monitor how many seeds germinate each day or every few days, but the exact time that seeds germinate is unknown. The only certain thing is that germination occurred between this and the prior inspection. Also, some seeds may not germinate within the experiment's duration, and the germination time is unknown. This phenomenon, called censoring, must be considered in the analysis to allow for valid inferences (Onofri et al., 2019). Time-to-event methods have been widely used in the agricultural or crop sciences to model the time-to-flowering (Ritz et al., 2010), the time-to-emergence (Onofri et al., 2010) or the time-to-germination (Onofri et al., 2011, 2018, 2019, 2022; Ritz et al., 2013) and have been proposed as one of the most suitable options for analyzing seed germination data

(Onofri et al., 2018). A time-to-event model can account for censoring and be used to analyze the time to germination and estimate the probability of germination for the seeds that did not germinate within the duration of the experiment. It can produce unbiased estimates of model parameters and their standard errors and is statistically robust and biologically comprehensible (Onofri et al., 2022). Several studies have shown that time-to-event methods lead to valid inferences and reliable hypothesis testing in many germination experiments (Hay et al., 2014; Ritz et al., 2013). Germination percentage, rate, and uniformity are the three critical traits of seed germination that can strongly influence the number, speed, and timing of successful seeds. A time-to-event model can, therefore, derive meaningful germination rate and uniformity data to predict the recruitment rate and the impact on species distribution and community structure more accurately.

Environmental conditions often vary along latitudinal and elevational gradients. In general, temperature decreases from low to high elevation and latitude; elevational and latitudinal gradients can be used as proxies to investigate plant species' responses to temperature (Wu et al., 2019). Our study aims to explore the research questions of (1) how temperature dependence of conifer seed germination varies inter- and intra-specifically from various locations and elevations and (2) whether the variation of germination traits relates to any climate factors at seed-collecting sites. Our hypotheses for the first question are: ( $H_{1a}$ ) The species (interspecific comparison) or population (intraspecific comparison) from higher elevations (cooler sites) will have lower optimal temperatures for seed germination and higher germination percentages at lower temperatures and vice versa because they have acclimated or adapted to local climatic conditions or habitats (Chamorro et al., 2018). ( $H_{1b}$ ) The central populations of each species' geographic distribution are expected to have the highest germination percentage in all temperatures because the environmental conditions are more favorable and stable at the center than at the edge of a species' geographic range (Abeli et al., 2014; Sagarin & Gaines, 2002). Therefore, the central population should have the most favorable physiological status, e.g., photosynthesis, which could produce more carbohydrates allocated to reproduction to overcome any environmental conditions within the upper and lower limits. The second research question hypothesis ( $H_2$ ) is that the variation of germination

traits relates to climate factors at seed-collecting sites because the germination responses vary based on physical, environmental, and geographic features where the seeds mature (Baskin & Baskin, 2014). We anticipate the results could provide valuable information for seed transfer, assisted migration, and forest management and predict population dynamics, community structure, and range shifts under climate warming.

## 4.2 Materials and Methods

### 4.2.1 Species and seed sources

We investigated four common conifer species in the Western United States: Lodgepole pine (*Pinus contorta* Dougl. ex. Loud.; PICO), Engelmann spruce (*Picea engelmannii* Parry ex Engelm.; PIEN), Ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.; PIPO), and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco; PSME; Figure 4.1). These species have high economic and ecological significance. They also have unique niches vulnerable to climate change, e.g., increasing frequency, extent, and severity of disturbance and changing climate that is faster than trees can adapt or migrate. Lodgepole pine has a broad ecological amplitude that grows under various climatic conditions (Burns & Honkala, 1990). Engelmann spruce grows in a humid climate with long, cold winters and short, cool summers. It occupies one of the highest and coldest forest environments in the Western United States (Burns & Honkala, 1990). Ponderosa pine is one of Western North America's most widely distributed pines, with seasonal rainfall deficiency in summer (Burns & Honkala, 1990). Douglas-fir naturally occupies a wide range of elevations and climatic conditions, extending from 19 to 55° N (Burns & Honkala, 1990). These four species occupy wide ranges in the Western United States, with varying climatic conditions (Table S4.1).

The seeds of these four species were donated by the Washington State Department of Natural Resources (Olympia, Washington, USA) and Silvaseed Company (Roy, Washington, USA), and each species was collected at different elevational ranges. Seed collection years vary by seedlots and range from 1976 to 2018. Seed collection and storage methods are unknown and may differ by seedlots or suppliers. Seed age was defined as the number of years from seed

collection to the seed germination test. Each seedlot was collected from an individual stand, and the geographic coordinates were assigned based on the latitudes and longitudes that match each seedlot's elevation and collection area in each seed zone (Tables 4.1 and S4.2). The elevational differences at which there are distinct adaptive genetic differences within the same species are 220m for Lodgepole pine, 370m for Engelmann spruce, 420m for Ponderosa pine, and 200m for Douglas-fir (Johnson et al., 2004). Therefore, we consider that seeds from the same location are genetically similar.

Table 4.1 Geographic coordinates, elevations, year collected, and seed age of *Pinus contorta* (PICO), *Picea engelmannii* (PIEN), *Pinus ponderosa* (PIPO), and *Pseudotsuga menziesii* (PSME) seeds acquired from the Washington State Department of Natural Resources (DNR) and the Silvasseed Company. †Seed age was defined as the number of years from seed collection to the seed germination test.

Species	Year Collected	Seed Age <sup>†</sup>	Elevation (m)	Latitude	Longitude
PICO	1979	41	975	48.86	-117.69
PICO	1982	38	1160	48.89	-117.67
PICO	1981	39	1370	48.88	-117.66
PICO	1979	41	1525	45.82	-117.97
PICO	1979	41	1705	47.86	-120.33
PIEN	1976	44	915	48.85	-119.59
PIEN	2009	11	1065	46.02	-121.62
PIEN	1978	42	1310	46.05	-117.45
PIEN	1978	42	1525	48.49	-117.53
PIEN	2001	19	1705	49.65	-120.15
PIPO	2018	13	760	45.96	-121.59
PIPO	1980	40	1065	38.82	-122.69
PIPO	2005	15	1220	44.24	-121.63
PIPO	1980	40	1370	39.90	-122.77
PIPO	1980	40	1525	39.91	-122.77
PSME	1995	25	150	43.89	-124.04
PSME	1995	25	460	43.95	-123.93
PSME	1985	35	760	41.00	-123.52
PSME	1996	24	1065	43.34	-122.87
PSME	1980	40	1525	39.91	-122.77

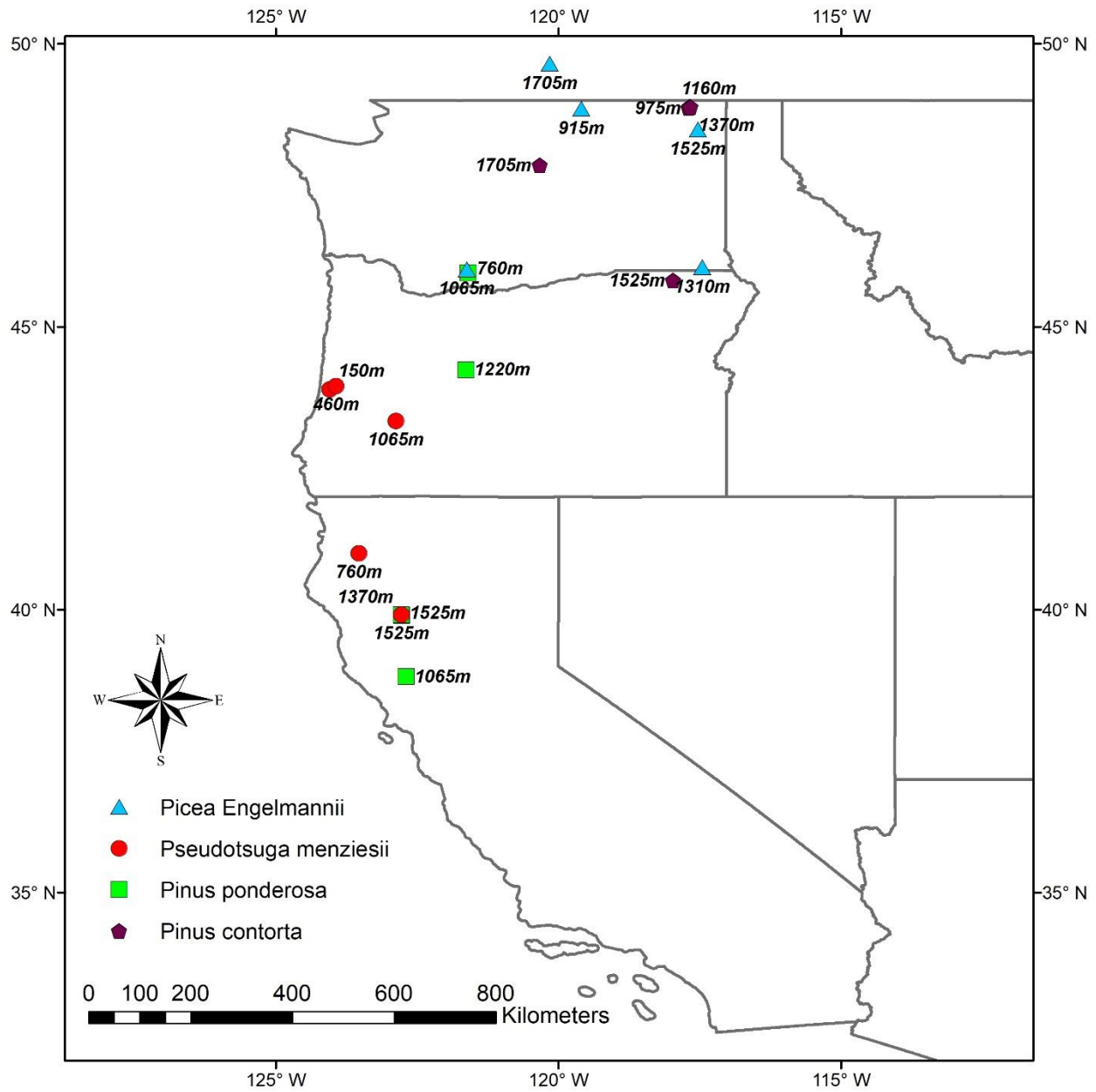


Figure 4.1 Seeds of *Pinus contorta*, *Picea engelmannii*, *Pinus ponderosa*, and *Pseudotsuga menziesii* were collected from different locations and elevations across the Western United States.

#### 4.2.2 Seed germination test

Seed germination tests were conducted from May to December 2020. We rinsed seeds with running water overnight and ran the test with wet Whatman® grade 1 cellulose filter

papers and Petri dishes in eight incubation temperatures from 5 to 40°C with an interval of 5°C. Eight units of G-1000 seed germinator (Convion, Winnipeg, Manitoba, Canada) were used to control temperatures and light (12 hours from 6 a.m. to 6 p.m., the light intensity is unknown). Deionized water was added to the Petri dishes to moisten the seeds as needed. Four Petri dishes of 25 seeds for each species (4 species), elevation (5 elevations), and temperature (8 temperatures) were used in each test round, and three test rounds (replicates) were conducted in different chambers. The four Petri dishes were considered subsamples to calculate the average of each round (true replicates). A seed was considered to have germinated when the radicle emerged and could be seen with the naked eye. Germinated seeds were counted and removed from Petri dishes every day for 30 days.

#### 4.2.3 Germination modeling

We applied a two-step thermal time-to-event model to our seed germination data. In the time-to-event model, the timing of germination within the seed population is considered to follow probability density functions. As a result, in the first step, we assumed the cumulative portion of germinated seeds ( $P$ ) increases over time ( $t$ ) according to a log-logistic cumulative probability (Eqn. 4.1).

$$P(t) = \frac{P_{max}}{1 + \exp\{a[\log(t) - \log(t_{50})]\}} \quad \text{Eqn. 4.1}$$

where  $t_{50}$  is the median germination time (unit: day),  $P_{max}$  is the maximum germination capability, and  $a$  is the slope at the inflection point. The three parameters describe the main features of germination, i.e., germination speed, capability, and uniformity, respectively. The goodness of fit for time-to-event models was checked graphically (Onofri et al., 2018). The fitted log-logistic cumulative probability function was used to derive the germination rate at the 50<sup>th</sup> percentile of the germinated fraction  $GR_{50}$  (a reciprocal of  $t_{50}$ , unit: number of seeds germinated per day). These values were used to parameterize the selected Gaussian function in the second step. We defined uniformity ( $U$ ; unit: day) as the time difference between the 25<sup>th</sup> ( $t_{25}$ ) and 75<sup>th</sup> ( $t_{75}$ ) percentile of the germinated fraction (Eqn. 4.2). Therefore,  $t_{25}$  and  $t_{75}$  were

also derived from the fitted log-logistic function to calculate the uniformity of seed germination.

$$U = t_{75} - t_{25} \quad \text{Eqn. 4.2}$$

A shorter period between  $t_{25}$  and  $t_{75}$  signifies a higher uniformity because it took less time to germinate 50% of seeds between 25% and 75% quartiles. As mentioned previously, parameter  $a$  is the slope at the inflection point of the log-logistic function. Therefore, a higher uniformity also means a steeper slope (higher  $a$  value).

Theoretically, the seed germination percentage reaches the maximum value at the optimal temperature and decreases when the temperature moves toward higher or lower limits. Therefore, bell-shaped and peak functions would be good candidates to fit germination data, and they have been widely applied in agricultural science to describe the rate of biological processes as a function of temperature (Archontoulis & Miguez, 2015). We used the Gaussian function (Eqn. 4.3) to fit the temperature response curves of seed germination.

$$G = G_{max} \exp\{-0.5[(T - T_o)/b]^2\} \quad \text{Eqn. 4.3}$$

where  $G$  is the response variable (germination percentage in % or  $GR_{50}$  in seeds day<sup>-1</sup>),  $T$  is the explanatory variable (temperature in °C),  $G_{max}$  is the maximum  $G$  value,  $T_o$  (optimal temperature in °C) is the position of the center of peak ( $G_{max}$ ),  $b$  (no unit) is the coefficient controlling the width of the bell shape, which represents an index of temperature range that seeds can germinate. The details of each variable and parameter in Eqn. 4.1, 4.2, and 4.3 are shown in Table 4.2. Time-to-event log-logistic and Gaussian functions were conducted in the R 4.1.2 programming language (R Core Team, 2021) with packages “drcSeedGerm” (Onofri et al., 2018) and “aomisc” (Onofri, 2020).

Table 4.2 Variables and parameters used in the log-logistic, uniformity and Gaussian functions and their descriptions.

Symbol	Unit	Description
<b>Log-logistic function [Eqn. 4.1]</b>		
$P(t)$	-	Cumulative portion of germinated seeds at time $t$ .
$t$	day	Time.
$t_{50}$	day	The day at the 50 <sup>th</sup> percentile of the germinated fraction.
$a$	-	The slope at $t_{50}$ .
$P_{max}$	-	Maximum germination capability.
<b>Uniformity [Eqn. 4.2]</b>		
$U$	day	Time difference between the 25 <sup>th</sup> and 75 <sup>th</sup> percentile of the germinated fraction
$t_{25}$	day	The day at the 25 <sup>th</sup> percentile of the germinated fraction.
$t_{75}$	day	The day at the 75 <sup>th</sup> percentile of the germinated fraction.
<b>Gaussian function [Eqn. 4.3]</b>		
$G$	%	The response variable: germination percentage.
	seeds day <sup>-1</sup>	The response variable: $GR_{50}$ .
$T$	°C	The explanatory variable: temperature.
$G_{max}$	%	Germination percentage: the maximum value for the expected response of $G$ .
	seeds day <sup>-1</sup>	$GR_{50}$ : the maximum value for the expected response of $G$ .
$b$	-	The shape parameter controls the width of the bell shape.
$T_o$	°C	The temperature at $G_{max}$ (optimal temperature).

#### 4.2.4 Statistical analysis

We implemented a one-way analysis of variance (ANOVA) and Tukey's honest significance test (HSD) to test the observed germination percentage (experimental data) among elevations in each species and temperature. A quadratic function was used to fit the coefficient of variances in each species and temperature. An analysis of covariance (ANCOVA) test was used to remove the effect of seed age as a covariable on seed germination traits. The ANOVA was also used to test the optimal temperature ( $T_o$ ), coefficient  $b$ , and maximum value ( $G_{max}$ ) extracted from the Gaussian function of germination percentage and  $GR_{50}$  among each species

and elevation. All analyses were conducted in the R 4.1.2 programming language (R Core Team, 2021).

The climate variables at the seed-collecting sites were calculated as averages from 1971 to 2000, which covered most of the seed-collection years and were obtained from ClimateNA (Wang *et al.*, 2016). Then, they were characterized using Principal Component Analysis (PCA). PCA was executed with the “prcom” function in the R 4.1.2 programming language (R Core Team, 2021) with the package “vegan” (Oksanen *et al.*, 2022). Twenty climate variables were used (Table S4.3). PCA reduced climate variables into several composite variables or principal components (PCs) that explained the most climate variations in seed collection sites. Interpretations of PCs were based on the magnitude and sign of each vector associated with individual climate variables. Variables with similar weights were deemed of similar importance. All PCs were examined, but only the first two were used to simplify the interpretation. The loadings of each climate variable of the first three components are shown in Table S4.4. The first three components explained 91.5% of the variance among different sites (Table S4.4). The climate at the seed collection sites varied with respect to mean annual temperature (MAT) and chilling (DD\_0) and growing degree days (DD5) (PC1; 65.1% of variance), and mean annual precipitation (MAP), mean May to September precipitation (MSP) and annual heat-moisture index (AHM) (PC2; 19.3% of variance; Figure S4.1). We interpreted that PC1 is related to temperature, and PC2 is associated with both temperature and precipitation. Lower PC1 scores are colder sites, and higher PC1 scores are warmer sites. Lower PC2 scores are warmer and wetter sites, and higher PC2 scores are colder and wet but relatively drier than sites with higher PC2 scores. We then performed simple linear regressions between PC scores and seed germination traits to test our hypotheses.

## 4.3 Results

### 4.3.1 Variations of seed germination traits

All four species produced the highest germination percentage at 20 or 25°C, indicating a similar optimal temperature range for germination (Figure 4.2). PIEN had a much higher

germination percentage (40.7 to 72.9%) than the other three species (1.7 to 24.3%) at 15°C (Figure 4.2 and Table S4.5). The germination percentage increased the most from 10 to 15°C for PIEN and from 15 to 20°C for PICO, PIPO, and PSME (Figure 4.2). Surprisingly, PIEN still had approximately 70% germination percentage at 30°C (Figure 4.2b and Table S4.5). None of the four species germinated at 40°C, while more PIEN and PIPO seeds than PICO and PSME seeds germinated at 5 and 10°C (Figure 4.2 and Table S4.5). Seeds from different elevations have significantly different germination percentages at most temperatures for each species (Figure 4.2 and Table S4.5). PSME seeds from elevations lower than 500m showed higher germination percentages than those higher than 500m at 25 and 30°C (Figure 4.2 and Table S4.5). However, most of the significant differences had no specific patterns at different elevations within the same temperature (Figure 4.2 and Table S4.5).

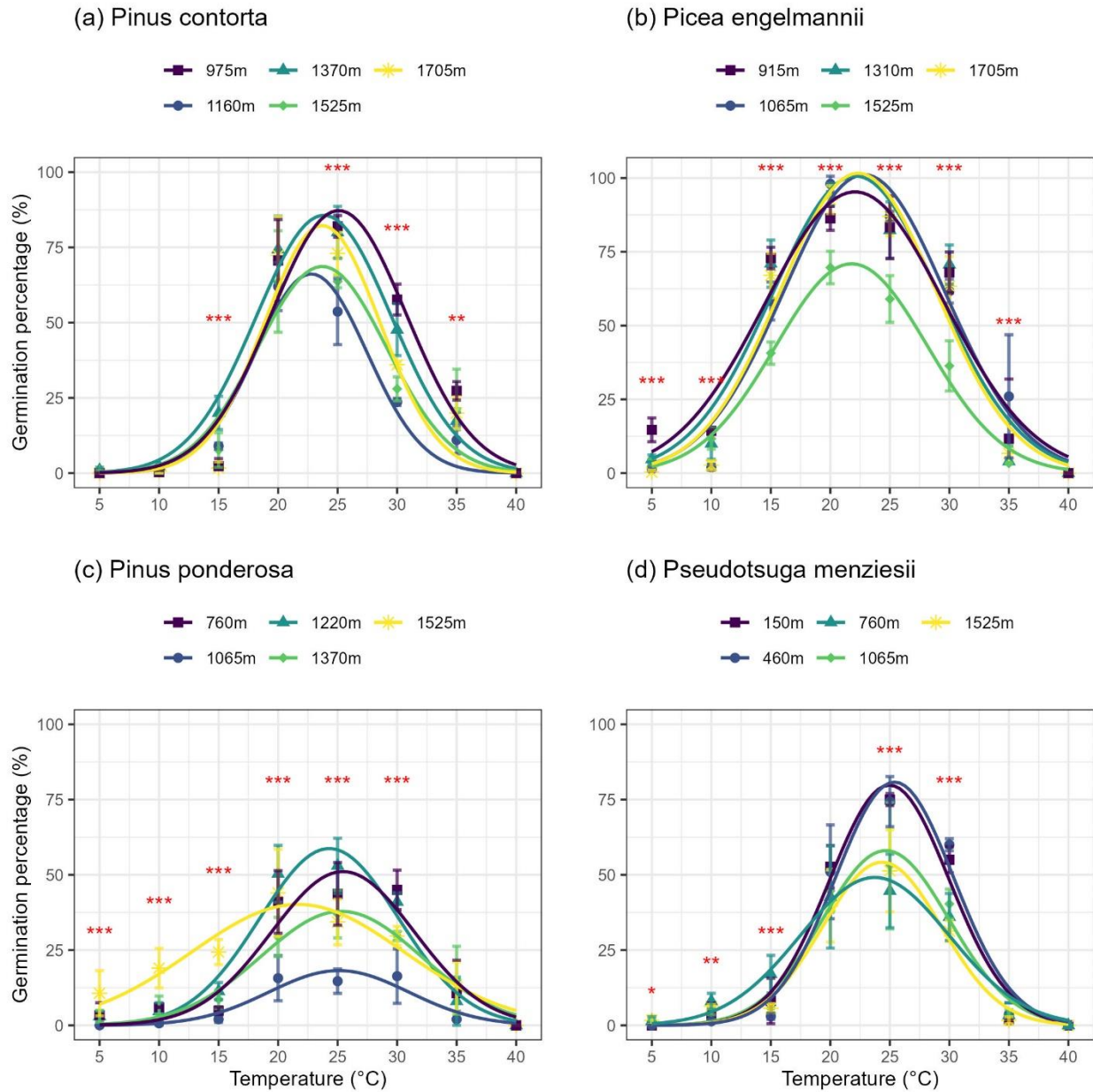


Figure 4.2 Observed seed germination percentage and fitted Gaussian temperature response curves of (a) *Pinus contorta* (PICO), (b) *Picea engelmannii* (PIEN), (c) *Pinus ponderosa* (PIPO), and (d) *Pseudotsuga menziesii* (PSME) from different elevations at different temperatures. Red asterisks represented the statistically different germination percentages among elevations at the same temperature. \*\*\*:  $p < 0.001$ ; \*\*:  $p < 0.01$ ; \*:  $p < 0.05$ .

The fitted germination curves showed that PIPO had extensive variation in  $T_o$ ,  $b$ , and  $G_{max}$  (Figure 4.2c and Table S4.6), and the other three species had more consistent optimal temperature and shape (Figures 4.2a, 4.2b, 4.2d, and Table S4.6). ANOVA results showed that PIEN had a significantly lower optimal temperature (22.2°C) than the other three species (PICO: 23.8°C; PIPO: 24.4°C; PSME: 24.6°C) ( $p < 0.001$ ; Table 4.3). PIEN and PIPO had greater  $b$  than PICO and PSME, which means the former two species had a wider temperature range for germination than the latter two species ( $p < 0.001$ ; Table 4.3). PIEN and PICO have higher  $G_{max}$  than PSME and PIPO ( $p < 0.001$ ; Table 4.3).  $T_o$  for seed germination from different elevations was significantly different in PICO ( $p < 0.05$ ) and PIPO ( $p < 0.05$ ), but no differences in PIEN ( $p = 0.544$ ) and PSME ( $p = 0.069$ ; Table S4.6). The shape coefficient  $b$  from different elevations was significantly different in PICO ( $p < 0.05$ ) and PIPO ( $p < 0.01$ ) but not in PIEN ( $p = 0.084$ ) and PSME ( $p = 0.066$ ; Table S4.6). The  $G_{max}$  from different elevations was significantly different in PIEN ( $p < 0.001$ ), PIPO ( $p < 0.001$ ), and PSME ( $p < 0.05$ ) but not in PICO ( $p = 0.078$  Table S4.6). Despite the  $T_o$ , shape coefficient  $b$  and  $G_{max}$  showing differences among elevations, the differences had no specific pattern or elevational gradient.

Table 4.3 The optimal temperature ( $T_o$ ), shape coefficient ( $b$ ), and maximum value at the optimal temperature ( $G_{max}$ ) for germination and  $GR_{50}$  of *Pinus contorta* (PICO), *Picea engelmannii* (PIEN), *Pinus ponderosa* (PIPO), and *Pseudotsuga menziesii* (PSME). The values are means of all elevations in each species  $\pm$  standard deviation. Different letters (a, b, and c) indicate significant differences between species (row-wise comparisons in germination and  $GR_{50}$ , respectively) by using Tukey's honest significance test (HSD). \*\*\*:  $p < 0.001$ ; \*\*:  $p < 0.01$ .

Germination					$GR_{50}$				
PICO	PIEN	PIPO	PSME	$p$ -value	PICO	PIEN	PIPO	PSME	$p$ -value
<b>Optimal temperature (<math>T_o</math>, °C)</b>									
23.8 $\pm$ 0.9 <sup>a</sup>	22.2 $\pm$ 0.8 <sup>b</sup>	24.4 $\pm$ 2.0 <sup>a</sup>	24.6 $\pm$ 0.9 <sup>a</sup>	<0.001 ***	24.1 $\pm$ 1.1 <sup>ab</sup>	23.1 $\pm$ 0.8 <sup>b</sup>	25.2 $\pm$ 3.4 <sup>a</sup>	22.4 $\pm$ 1.7 <sup>b</sup>	<0.01 **
<b>Shape coefficient (<math>b</math>)</b>									
5.2 $\pm$ 0.5 <sup>b</sup>	7.0 $\pm$ 0.5 <sup>a</sup>	6.4 $\pm$ 1.7 <sup>a</sup>	5.3 $\pm$ 0.7 <sup>b</sup>	<0.001 ***	6.1 $\pm$ 1.0 <sup>b</sup>	7.2 $\pm$ 0.7 <sup>b</sup>	9.7 $\pm$ 2.7 <sup>a</sup>	9.8 $\pm$ 1.2 <sup>a</sup>	<0.001 ***
<b>Maximum value at optimal temperature (<math>G_{max}</math>)</b>									
0.79 $\pm$ 0.12 <sup>a</sup>	0.91 $\pm$ 0.10 <sup>a</sup>	0.42 $\pm$ 0.14 <sup>c</sup>	0.65 $\pm$ 0.17 <sup>b</sup>	<0.001 ***	0.22 $\pm$ 0.04 <sup>a</sup>	0.22 $\pm$ 0.03 <sup>a</sup>	0.09 $\pm$ 0.03 <sup>b</sup>	0.09 $\pm$ 0.02 <sup>b</sup>	<0.001 ***

The coefficients of variance (CV) of seed germination percentages of each species in all experimental rounds at different temperatures showed that variation increased when the temperatures shifted from optimum toward upper or lower limits (Figure S4.2). The fitted quadratic curves showed the variation was slightly higher at the lower-limit temperature than at the higher-limit temperature (Figure S4.2). We excluded the data from 5 and 40°C because there was no or very low germination.

#### 4.3.2 Modeling seed germination speed ( $GR_{50}$ ) and uniformity ( $T_{75} - T_{25}$ ) with time-to-event model and Gaussian function.

The results showed that the three-parameter log-logistic function fits the germination data well (Figure 4.3). We then derived  $GR_{50}$ ,  $T_{25}$ , and  $T_{75}$  from all fitted log-logistic functions and then fitted  $GR_{50}$  to the Gaussian function (Eqn. 4.3, Table 4.2) to estimate the  $T_o$ ,  $b$ , and  $G_{max}$  of  $GR_{50}$ .

The  $GR_{50}$  of PIPO and PSME had low values (mostly below 0.1, Figure 4.4c and 4.4d). ANOVA results showed that PIPO had a significantly higher optimal temperature (25.2°C) than the other three species (PICO: 24.1°C; PIEN: 23.1°C; PSME: 22.4°C) ( $p < 0.01$ ; Table 4.3). PIPO and PSME had greater  $b$  than PICO and PIEN ( $p < 0.001$ ; Table 4.3), meaning PIPO and PSME had a broader range of temperatures that  $GR_{50}$  was relatively higher. PICO and PIEN have much higher  $G_{max}$  than PSME and PIPO ( $p < 0.001$ ; Table 4.3).

$T_o$  for  $GR_{50}$  from different elevations was significantly different in PIEN ( $p = 0.051$ ) and PSME ( $p < 0.05$ ), but no differences in PICO ( $p = 0.541$ ) and PIPO ( $p = 0.949$ ; Table S4.7). The shape coefficient  $b$  from different elevations was significantly different only in PIPO ( $p < 0.05$ ; Table S4.7). The  $G_{max}$  of  $GR_{50}$  from different elevations was significantly different in PIEN ( $p = 0.054$ ) and PSME ( $p < 0.001$ ) but not in PICO ( $p = 0.125$ ) and PIPO ( $p = 0.548$  Table S4.7). Despite the  $T_o$  and  $G_{max}$  for  $GR_{50}$  of PIEN showing differences among elevations, the differences had no specific pattern or elevational gradient.

The results showed that the uniformity of PIEN and PICO in all experimental rounds at different temperatures decreased when the temperatures shifted from optimum toward upper or lower limits (Figures S4.3a and S4.3b). The ANOVA results showed that uniformity is significantly lower at 30°C than at other temperatures in both PIPO ( $p < 0.01$ ) and PSME ( $p < 0.001$ ; Figures S4.3c and S4.3d).

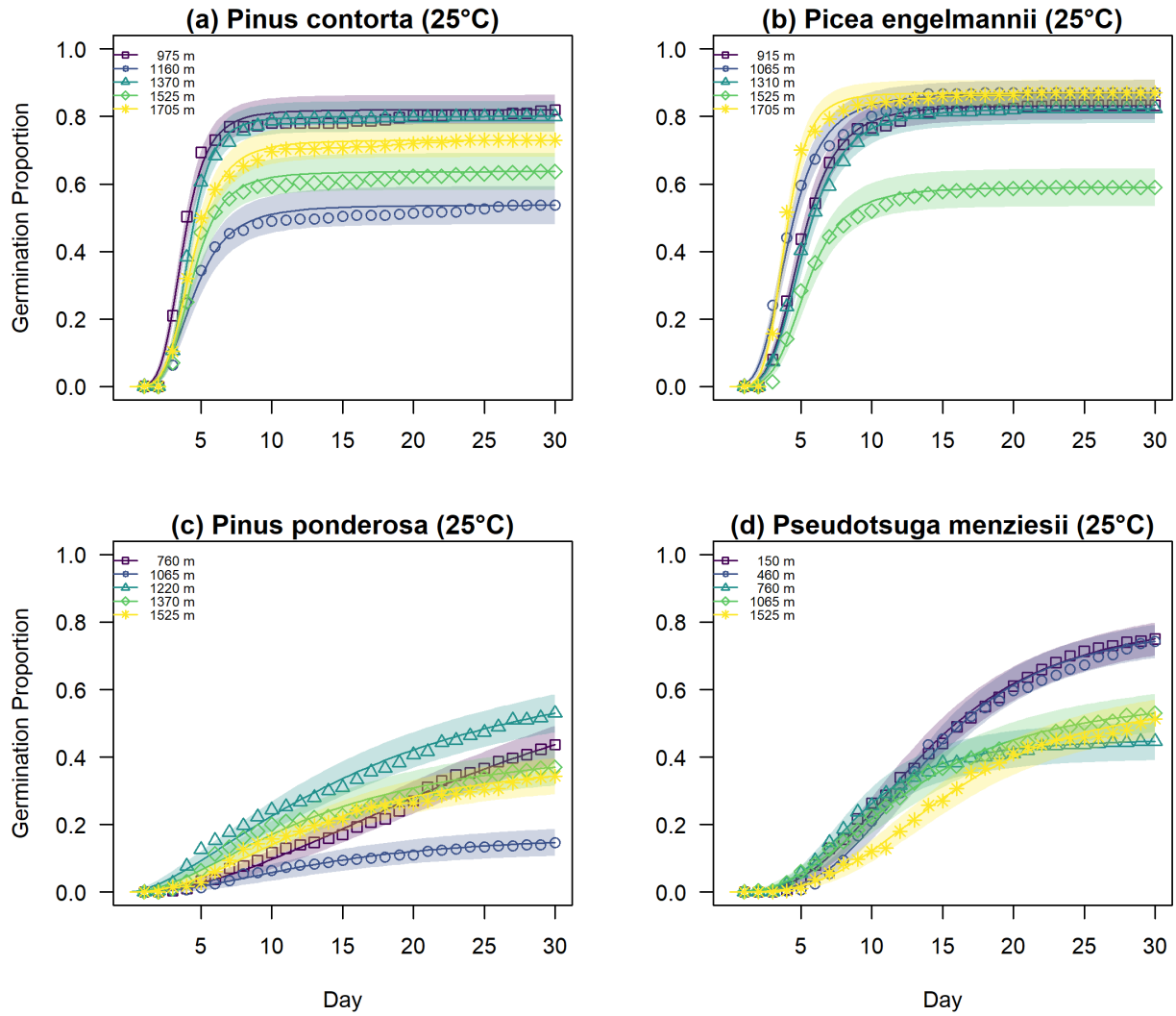


Figure 4.3 An example of the three-parameter log-logistic function fitting the accumulated numbers of seed germinated over 30 days for (a) *Pinus contorta* (PICO), (b) *Picea engelmannii* (PIEN), (c) *Pinus ponderosa* (PIPO), and (d) *Pseudotsuga menziesii* (PSME) at 25°C.

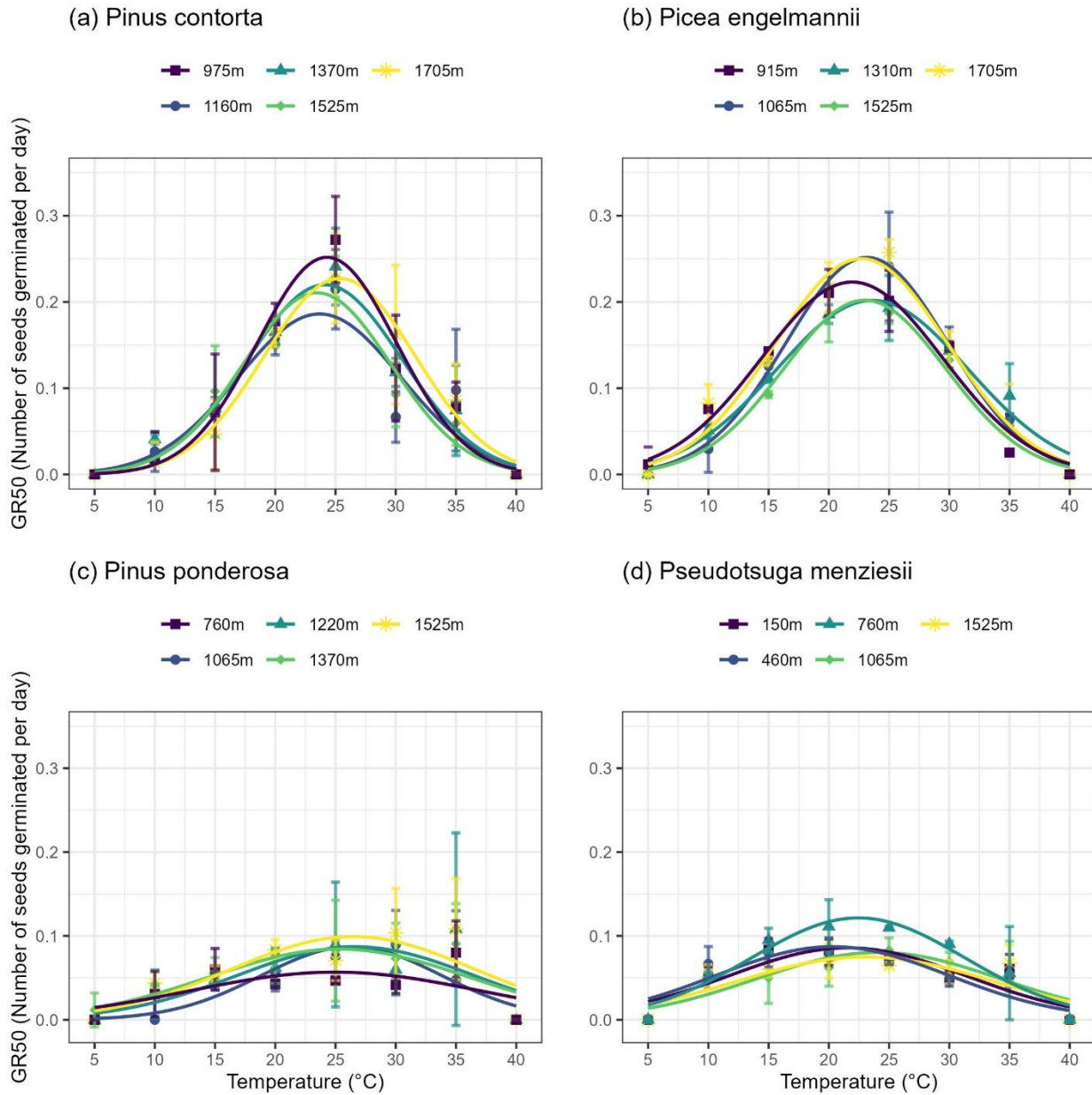


Figure 4.4  $GR_{50}$  temperature response curves of (a) *Pinus contorta* (PICO), (b) *Picea engelmannii* (PIEN), (c) *Pinus ponderosa* (PIPO), and (d) *Pseudotsuga menziesii* (PSME) from different elevations.

#### 4.3.3 Climatic effects on seed germination traits

The results showed seeds from colder habitats had a lower  $T_o$  and higher  $G_{max}$  and vice versa, regardless of species, and each species grouped well (Figures 4.5a and 4.5e). The trends in PC2 scores showed that seeds from warmer and wet habitats had a higher  $T_o$  and  $G_{max}$  and

lower  $b$  and vice versa, regardless of species, and each species did not group very well (Figures 4.5b, 4.5d, and 4.5f). However, there were only a few intra-species trends along climate-related PC1 and PC2 (Figure S4.4), e.g., PIPO from cooler habitats had higher  $G_{max}$ , PIEN and PSME had smaller  $b$  at cooler habitats (regression lines in Figure S4.4a), PIEN from wet and relatively cooler sites had larger  $b$ , and PSME from wet sites that is relatively cooler had lower  $T_o$  and  $G_{max}$  (regression lines in Figure S4.4b). We also examined the relationships between observed germination percentages at different temperatures and PC scores within individual species. The results showed that the observed seed germination percentage only had environmental gradients along PC1 in a few PIPO and PSME temperatures (Table S4.8 and Figures S4.5 and S4.6) and in more species and temperatures along PC2 (Table S4.8, Figures S4.7, S4.8, and S4.9). Negative and positive slopes mean germination percentages decrease and increase, respectively, with increasing PC1 or PC2 scores.

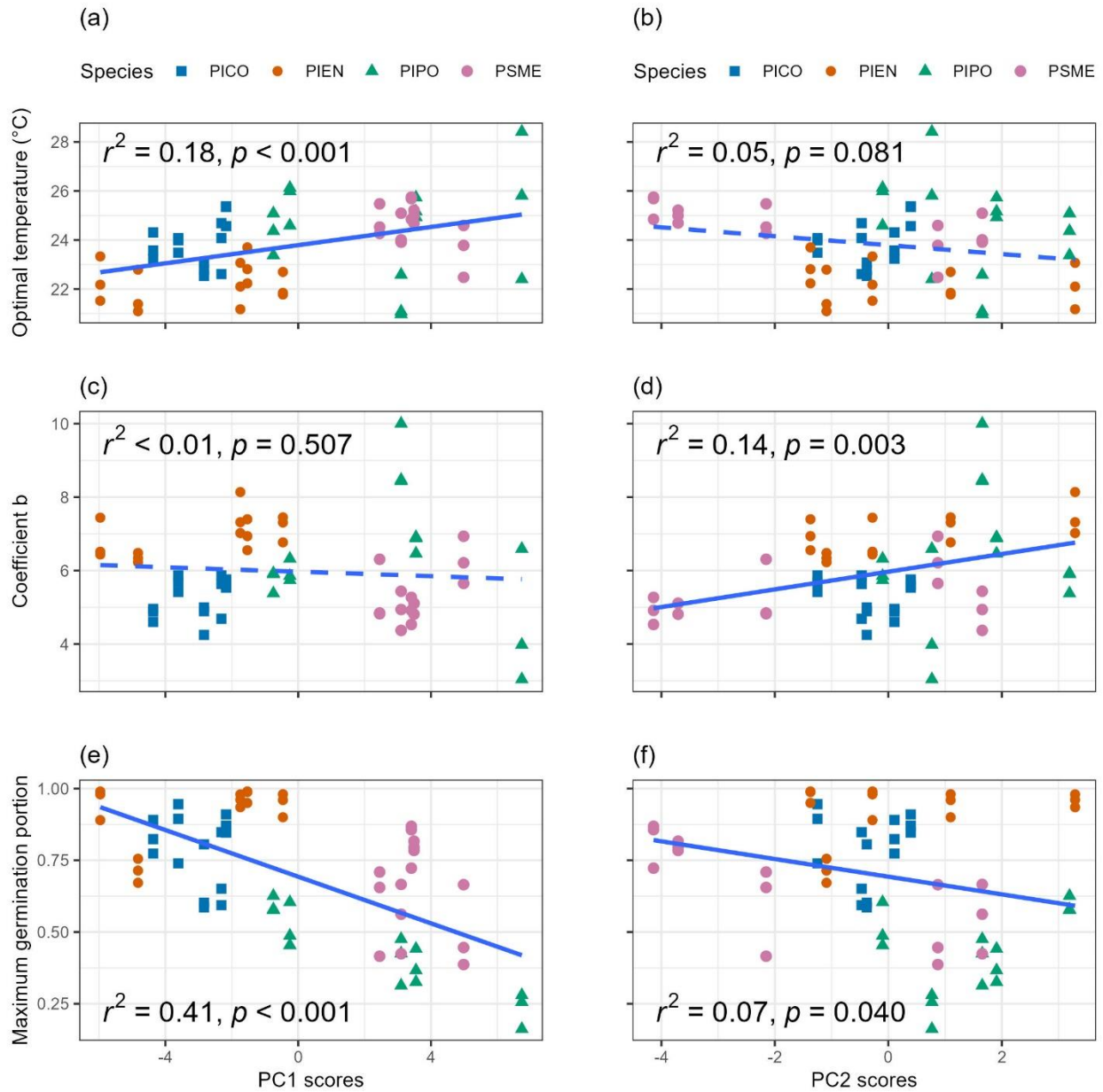


Figure 4.5 Inter-species relationships between PC1 scores and optimal temperature (a), coefficient  $b$  (c), and maximum germination portion (e); between PC2 scores and optimal temperature (b), coefficient  $b$  (d), and maximum germination portion (f) of *Pinus contorta* (PICO, blue squares); *Picea engelmannii* (PIEN, red circles); *Pinus ponderosa* (PIPO, green triangles); *Pseudotsuga menziesii* (PSME, pink circle). Dashed line: non-significant trend. Solid lines: significant trends. Lower PC1 scores are colder sites, and higher PC1 scores are warmer sites. Lower PC2 scores are warmer and wetter sites, and higher PC2 scores are colder and wet but relatively drier than sites with lower PC2 scores.

## 4.4 Discussions

### 4.4.1 How does the temperature response of seed germination vary among four conifer species?

Current species distributions are presumed to represent an acclimation or adaptation to the environment in which they live. PIEN occupies one of the highest and coldest forest environments in the Western United States. Its viable seeds that survive over winter usually germinate following snowmelt when seedbeds are moist and air temperature is at least 7°C (Burns & Honkala, 1990). Our results also show that PIEN has a higher germination percentage than the other three species at lower temperatures (10 to 15°C, Figure 4.2; Table S4.5). The other three species have overlapping natural habitats that are widely distributed and include a wide span of elevations and climatic conditions (Burns & Honkala, 1990). It seems they also have similar and relatively higher germination percentages across a wide range of temperatures at 20, 25, and 30°C (Figures 4.2a, 4.2c, and 4.2d). In addition, the  $T_o$  of PIEN is lower than that of the other three species (Table 4.3). These results suggest that species have acclimated or adapted to the environment they occupy. PICO and PSME have lower  $b$  than PIEN and PIPO (Table 4.3). Interestingly, PICO is a species with a broad ecological amplitude (Burns & Honkala, 1990) but does not have a wider temperature range in which seeds can germinate for the seed tested (Table 4.3). The possible reason is that seed sources of PICO in our study came from a relatively narrower geographic range that did not cover the whole range of the species (Figure 4.1), had limited climatic variability (Figure S4.1), and/or may not represent all ecotypes. On the other hand, PIEN has a higher germination percentage at 30°C compared to the other species tested (Figure 4.2 and Table S4.5) and a wider temperature range with seeds germinating (Table 4.3). However, the range of mean annual temperatures that PIEN grows is narrow (Burns & Honkala, 1990). The results suggest that seed germination of PIEN may benefit from warming. Wu et al. (2019) had similar findings that species from higher elevations are more sensitive to temperature, and warming has a greater effect on germination speed and percentage.

As found by a recent meta-analysis, temperature and germination time also have a positive relationship (Fernández-Pascual et al., 2019). The mean time to germination is longer at lower and higher temperatures and shorter at optimal temperatures (Bonner & Karrfalt,

2008). We use the germination rate at the 50<sup>th</sup> percentile of the germinated fraction ( $GR_{50}$ ) to represent the germination time. Theoretically, the faster the germination rate, the shorter the germination time. Our results also show that the  $GR_{50}$  of all species is faster at optimal temperatures and slower at low and higher temperatures (Figure 4.4). In addition, the estimates of the  $GR_{50}$  coefficient ( $b$ ) are also greater than those of the germination percentage of all species, meaning climatic warming may have less effect on  $GR_{50}$  than the germination percentage.  $GR_{50}$  of PIPO and PSME had smaller values, and this is also demonstrated by Figures 4.3c and 4.3d, where PIPO and PSME had fewer seeds germinate simultaneously (gradual slopes in Figures 4.3c and 4.3d), even though the temperature (25°C) is close to optimum. This may be related to the seed dormancy. PIPO and PSME have physiological dormancy that must be broken before sowing them (Baskin & Baskin, 2014), and pre-germination treatment of PIPO and PSME seeds strongly influences their subsequent response to various germination conditions (Bonner & Karrfalt, 2008). Dormancy may explain why the  $GR_{50}$  was low ( $<0.1$ ; Figure 4.4c and 4.4d). Another possibility is that the  $GR_{50}$  is derived from the time-to-event log-logistic model. If only a few seeds germinated, uniformity might also be biased by low germination, and the ways we handle germination modeling will have considerable differences in  $GR_{50}$ , section 4.4.3. The breadth of the optimal temperature range for germination can change as the seed matures and ages and as dormancy is imposed or alleviated (Alvarado & Bradford, 2002; Simons, 2011; Steadman et al., 2004). However, we found that seed age did not affect the optimal temperature ( $p=0.307$ ) and the temperature range that seeds can germinate ( $p=0.44$ ; Table S4.9), but we did not test seed dormancy in our study.

When we look into the climate at different sites, the first two PCs explain 84.4% of the climatic variations across the sites (Figure S4.1). PC1 is related to temperature, and PC2 is associated with both temperature and precipitation. In other words, temperature and precipitation combined explain more than 80% of the climate difference across sites. The seed germination traits show some trends across PC1 and PC2 scores at different sites regardless of species (Figure 4.5), and they support our hypothesis that species or populations from colder sites have low optimal temperatures and vice versa ( $p<0.001$  and  $p=0.081$ ; Figures 4.5a and 4.5b). Cochrane et al. (2014) also found a significant correlation between optimal germination

temperatures and mean annual temperatures at seed sources across the species. It reinforces that seeds from different locations have been acclimated or adapted to their habitats, and the germination responses vary based on physical, environmental, and geographic features where the seeds mature (Baskin & Baskin, 2014; Liu & El-Kassaby, 2015; Stoehr et al., 1998).

#### 4.4.2 How does the temperature response of seed germination vary among populations within the same species collected from various locations and elevations?

Based on our hypothesis, we expect variations in seed germination traits within populations, which reflect the climatic gradients of seed origin. Some studies have observed that species have substantial among-population variation in environmental sensitivity for germination success (Cochrane et al., 2014; Cochrane, et al., 2015a; Walter et al., 2020). Similarly, we find that seeds from different elevations showed significant differences in germination percentages at most temperatures for each species (Figure 4.2 and Table S4.5) and also in optimal temperature ( $T_o$ ), the temperature range that seeds can germinate ( $b$ ), and germination percentage at optimal temperature ( $G_{max}$ ) for some species (Table S4.6). However, the significant differences have no specific patterns along the elevational gradient, neither at different temperatures nor at different elevations within the same temperature. This suggests that elevation alone is not a reliable predictor of variation among populations, especially when the elevation gradient is spread across a wide geographic range, similar to the findings of Wu et al. (2019). Therefore, we use PCA to characterize the climate variables at seed-collecting sites to see whether climate-related PC scores can explain variations among populations better. If populations of a species acclimate to their environment, their seed germination may respond differently to environmental changes because germination responses are related to the climate where the seeds mature (Baskin & Baskin, 2014; Liu & El-Kassaby, 2015; Stoehr et al., 1998). That is, the population from higher elevations (cooler sites) will have higher germination percentages at lower temperatures and vice versa. In our PCA results, this is only true in some cases (Table S4.8 and Figures S4.5 to S4.9) and mostly related to the precipitation and heat-moisture index (PC2), suggesting that the temperature-only component (PC1) may not explain the relationship between the environmental gradient and seed germination percentage well,

and both temperature and precipitation should be considered. In reality, seeds experience varying levels of diurnal temperature fluctuation at different elevations. We only tested constant temperatures in growth chambers. It is worth exploring how temperature fluctuations will affect the intraspecific temperature response of seed germination in the future.

According to our first hypothesis, if the temperature is lower than the optimum, the population from the higher elevation should have a higher germination percentage and vice versa. Only PSME in PC2 (Table S4.8 and Figure S4.9) matches our first hypothesis. PIPO in PC1, PICO, and PIEN in PC2 partially match our first hypothesis (Table S4.8 and Figures S4.5, S4.7, and S4.8). Therefore, our first hypothesis is not fully supported due to the divergent results in each species (Table S4.8 and Figures S4.5 to S4.9).

We do not find any central populations (i.e., the middle elevation of tested seed sources in our study) with higher germination percentages at all temperatures. We reject the second hypothesis (Table S4.5). Wu et al. (2019) also found central populations do not have the advantage, and the germination percentage was even lower than marginal populations. Abeli et al. (2014) stated that abundant center hypothesis expectations were also not met in most cases in their study and that population and plant size met the abundant center hypothesis more often. It suggests that seed germination traits do not benefit from a stable environment and more resources in the central population. Instead, they are correlated with the environment where the seeds mature (Baskin & Baskin, 2014; Cochrane et al., 2014; Gao et al., 2021; Haasis & Thrupp, 1931; Liu & El-Kassaby, 2015; Roche, 1969; Stoehr et al., 1998).

Environmental gradients also do not appear to be reliable predictors of variation among populations, and responses are often individualistic (Cochrane et al., 2015a). Of the results that do not support our first hypothesis, some of them show that seeds from cooler sites consistently had higher germination percentages at both higher and lower temperatures (Figures S4.5 and S4.8) or seeds from cooler sites had lower germination percentages at lower temperatures (Figure S4.7). One explanation is that seed sources in our study came from a relatively narrower geographic range that did not cover the whole range of the species. It is also possible that some other factors affect the seed germination and dilute the effect of elevation, for example, seed ages. We found that the seed age or duration in storage since collection

might contribute to no specific patterns along the elevational gradient because the germination percentage decreased with increasing age (Figure S4.10a). The ANCOVA results showed that the significance of  $T_o$ ,  $b$ , and  $G_{max}$  among species did not change after considering seed ages. However, it revealed that seed age did not affect  $T_o$  ( $p=0.307$ ) and  $b$  ( $p=0.44$ ) but significantly affected  $G_{max}$  ( $p<0.001$ ; Table S4.9). Anderegg (2023) had similar opinions that several reasons may explain inconsistent and weak trait-climate relationships, including (1) incomplete sampling niche; (2) confounding factors that are difficult to disentangle geographically; (3) micro- and macro-climate variation decouples the environment that sampled individual actually experience from environmental predictor used in the analysis; and (4) nonadaptive trait variation.

In addition, we observed that the germination percentage and uniformity had smaller variations near optimal temperatures and larger variations when the temperature moved toward upper or lower limits in each species (Figure S4.2). Arnold et al. (2022) had similar observations and found that germination and reproductive traits showed the best performance at intermediate temperatures. In the optimal temperature range, seed germination is relatively consistent among populations, as the conditions are generally favorable for growth and development. In suboptimal temperature ranges, there is greater variation in seed germination among populations, as the conditions are less favorable for growth. At temperature limits, such as the minimum or maximum temperature tolerances of species in our study, there is even more significant variation in seed germination among populations. This is because the conditions are more extreme, and fewer populations can tolerate them. It is also possible that there are many zero germination percentages at extreme temperatures, resulting in a higher coefficient of variation. We cannot confirm whether it has ecological significance or is a mathematical artifact in our study. For survival in the natural environment, plants have evolved a “bet-hedging” strategy where individual variation is high and a range of phenotypes is produced. When faced with unpredictable environmental conditions, fluctuation in seed behavior is a beneficial trait that allows plant species to survive (Sharma & Majee, 2023). In summary, the interspecific seed germination traits have an optimal temperature that suits their past natural habits (Figure 4.5). However, the intraspecific germination traits among various

sites did not match our first hypothesis well (Table S4.8 and Figures S4.4 to S4.9). Anderegg (2023) had similar findings highlighting how divergent results of trait-environment relationships within species can be, but more clearly interspecific trait-climate relationships can be seen.

#### 4.4.3 Limitations of the study and future direction

Due to the availability of seed sources, our study was not designed to differentiate the genetics (G), the environment (E), and their interactions with seed sources. Instead, we focus on the environmental conditions of seed-collecting sites and how they relate to the intra- and inter-specific variations of seed germination response to different temperatures, which provides valuable information for seed transfer, assisted migration, and forest management. As we show in sections 3.1 and 3.2, the observed germination percentage,  $GR_{50}$ , and optimal temperatures varied significantly between locations with different elevations, but the variations were not explained by the elevational gradient alone. The mean annual temperature often decreases as the elevation increases, meaning locations at higher elevations are colder. To test our hypothesis that germination responses to temperature may vary among populations across elevations, we used seeds that were not only from different elevations but also across a wide range of the Western United States (Figure 4.1), making temperature confounded with many other factors. To account for this, we used a PCA to characterize the climatic differences among these sites and correlate climate with seed germination traits. However, additional factors relating to seed quality created other errors; for example, seed age ranged from two years to more than 40 years old. PIPO is one example where seed age was a confounding factor. We find that seeds from cooler sites had higher or equal germination percentages at all temperatures, but seeds from these sites were much younger than seeds from all the others (Table S4.2). Due to the small sample sizes of different seed ages and elevations of individual species, we could not analyze the seed age effect on the individual species from different elevations. Seed ages are related to the seed germination percentages (Figure S4.10a), and additional variation in seed quality related to collecting, handling, and storage also cannot be quantified. All of these factors may dilute the environmental and elevational trends. Understanding this variation is important for predicting and managing plant population dynamics and for conserving plant

diversity in the face of environmental change. However, many other factors impact seed germination traits related to unknown seed quality characteristics. We recommend seed collection specifically for research to minimize other unwanted factors in the future.

The  $GR_{50}$  is derived from the time-to-event model, and the advantage of this model is to comply with the typical traits of the observed data, such as censoring (Onofri et al., 2022) to take the germination capability into account. However, this also creates another issue, which is the germination capability can still be high if only a few seeds germinate. How low germination is treated in the time-to-event model will greatly impact results, including  $GR_{50}$  estimates. For example, only very low germination was observed at 5 and 35°C in our study. If these low numbers of germinated seeds are assumed to be either the full range or the lower asymptote of the log-logistic function, the resulting  $GR_{50}$  estimation will be significantly different. Similarly, the uniformity will be very high if only one or two seeds in total are germinated at the same time, even if the temperature is not optimal and the total germinated proportion is low. In cases of physiological dormancy, the maximum germination percentage and  $GR_{50}$  estimated by the time-to-event model may be unreliable. For example, we only see one-third of the seeds germinated in Figures 4.3c and 4.3d, but the germination capacity estimated by the time-to-event model reaches much higher values, impacting the  $GR_{50}$  estimations. This explains the low values and variations in temperature response for  $GR_{50}$  in PIPO and PSME (Figure 4.4).

Foresters have long known that using locally produced seeds promotes the success of reforestation from seeds (Johnson et al., 2004). However, because of rapid climate change, people can no longer assume that locally sourced seeds are always the best or appropriate option (Havens et al., 2015). Plants are responding to changing climates through plasticity, adaptation, and migration, and knowing how this may influence seed-sourcing decisions is essential for successful restoration in the future. Temperature is one of the most critical environmental factors that regulate seed germination, but not the only factor. Our results suggest that temperature and precipitation combined can better predict the variation within the species. Elevation alone is not a strong proxy to estimate seed germination traits as it does not account for latitudes, aspects, or other site-specific conditions; for example, the climate at 1000m in different geographical regions could vary significantly. Therefore, developing

consistent tools for characterizing the climate of seed-collecting sites that regulate seed germination is essential to help with seed transfer decisions. Continued collection of seeds from diverse environments and studying abiotic and biotic effects on seed germination are critical to improving our understanding of how the early stage of plant life will respond to climate change. This information can help identify the most crucial factors for species regeneration and aid in seed transfer, assisted migration, and designing effective refugia as climate change progresses.

#### 4.5 Conclusions

Germination is highly sensitive to climatic conditions and may vary among species and populations. Our results illustrate the temperature dependence of seed germination across a wide range of temperatures in four conifer species from different locations and elevations. They show that seeds from different species and from different elevations within the same species had different germination traits correlated with their ecological niche. However, the germination traits did not conform with the environmental gradients that we hypothesized. We found that the variations of germination traits are larger when the temperature approaches the upper and lower biological limits of germination. While we are unable to pinpoint the reasons for intraspecific variation of seed germination traits, the variations we found may represent genetic diversity, increase species resilience, improve the chance of species persistence under climate change, and mitigate species' vulnerability to changing climate. Seed germination traits have been neglected in vegetation and ecophysiological science compared with seed morphological traits. Determining the temperature dependence of seed germination from different provenances and locations is essential to evaluate species' response to temperature for predicting and mitigating the impacts of climate change on plant populations and ecosystems. We anticipate applying these understandings to predict population dynamics and range shifts under climate warming and expect this information can also help with adjusting forest management practices, seed transfer, and assisted migration.

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Supplementary materials

Table S4.1. Climatic conditions of nature habitats (Burns and Honkala, 1990) and seed collection sites (Wang *et al.*, 2016) of each species. MAT: mean annual temperature; MAP: mean annual precipitation.

Species	<i>Picea engelmannii</i>		<i>Pinus contorta</i>		<i>Pinus ponderosa</i>		<i>Pseudotsuga menziesii</i>	
<b>Nature habitats</b>								
Temperature range	4.	MAT: -1 to 2°C	3.	Min T: -57 to 7°C	3.	MAT: 5 to 10°C	1.	Mean Jan T: -9 to 3°C
	5.	Mean Jan T: -12 to -7°C	4.	Max T: 27 to 38°C	4.	Mean Jul-Aug T: 17 to 21°C	2.	Mean Jul T: 7 to 30°C
	6.	Mean Jul T: 4 to 16°C						
MAP range	610 to 4060 mm		250 to 500 mm		280 to 1750 mm		360 to 3400 mm	
<b>Seed collection sites</b>								
MAT	915m	6.2°C	975m	6.1°C	760m	7.5°C	150m	11.2°C
	1065m	6.3°C	1160m	5.4°C	1065m	13.7°C	460m	11°C
	1310m	7.4°C	1370m	4.7°C	1220m	7.1°C	760m	12.6°C
	1525m	3.4°C	1525m	5.6°C	1370m	11.2°C	1065m	10.3°C
	1705m	1.7°C	1705m	3.4°C	1525m	10.8°C	1525m	10.8°C
MAP	915m	425 mm	975m	885 mm	760m	1297 mm	150m	2208 mm
	1065m	1566 mm	1160m	990 mm	1065m	1513 mm	460m	2482 mm
	1310m	732 mm	1370m	1100 mm	1220m	580 mm	760m	1675 mm
	1525m	1175 mm	1525m	1180 mm	1370m	880 mm	1065m	2053 mm
	1705m	685 mm	1705m	962 mm	1525m	891 mm	1525m	891 mm

Table S4.2. *Pinus contorta* (PICO), *Picea engelmannii* (PIEN), *Pinus ponderosa* (PIPO), and *Pseudotsuga menziesii* (PSME) seeds acquired from the Washington State Department of Natural Resources (DNR) and the Silvaseed Company.

Lot #	Quantity (gram)	Species	Seed zone / Elevation (m)	Old zone / collection area	Year collected	Test year / germination %	Source
72	10	PIEN	PIEN 05 / 915	611	1976	2014 / 75.25	DNR
1268	10	PIEN	PIEN 04 / 1065	652 / Trout Lake	2009	2010 / 98.75	DNR
1171	10	PIEN	PIEN 09 / 1310	La Grande Ranger District	1978	2008 / 93.25	DNR
1174	10	PIEN	PIEN 08 / 1525	821	1978	2008 / 80.5	DNR
1079	10	PIEN	PIEN 05 / 1705	N49'39" W120'09" McNulty Cr BC	2001	2014 / 92.25	DNR
1092	10	PICO	PICO 15 / 975	Plum Cr Timber Co Unit 21	1979	2014 / 90.75	DNR
1094	10	PICO	PICO 15 / 1160	Plum Cr Timber Co Unit 21	1982	2014 / 83	DNR
1095	10	PICO	PICO 15 / 1370	Plum Cr Timber Co Unit 21	1981	2014 / 90	DNR
948	10	PICO	PICO 17 / 1525	863 / Oregon	1979	2005 / 92	DNR
1114	10	PICO	PICO 12 / 1705	621 / Baldy Mtn 27N 20E	1979	2014 / 92	DNR
R18008	300	PIPO	652 / 760	Trout Lake WA	2018	2019 / 79	Silvaseed
R80008	300	PIPO	380 / 1065	Middletown CA	1980	2019 / 51	Silvaseed
R05002	300	PIPO	675 / 1220	Sisters OR	2005	2017 / 92	Silvaseed
R80218	300	PIPO	371 / 1370	Pollard Corral CA	1980	2019 / 73	Silvaseed
R80219	300	PIPO	371 / 1525	Pollard Corral CA	1980	2017 / 85	Silvaseed
B18005	100	PSME	062 / 150	Florence OR	1995	2018 / 87	Silvaseed
B18007	100	PSME	062 / 460	Florence OR	1995	2018 / 93	Silvaseed
B89016	100	PSME	303 / 760	Grouse Mountain CA	1985	2017 / 75	Silvaseed
B96036	100	PSME	491 / 1065	Glide OR	1996	2016 / 82	Silvaseed
R80105	100	PSME	371 / 1525	Pollard Corral CA	1980	2014 / 88	Silvaseed

Table S4.3 The climate variables that were used in the Principal Component Analysis.

<b>Abbreviation</b>	<b>Description</b>
MAT	mean annual temperature (°C)
MWMT	mean warmest month temperature (°C)
MCMT	mean coldest month temperature (°C)
TD	temperature difference between MWMT and MCMT, or continentality (°C)
MAP	mean annual precipitation (mm)
MSP	May to September precipitation (mm)
AHM	annual heat-moisture index $(MAT+10)/(MAP/1000)$
SHM	summer heat-moisture index $((MWMT)/(MSP/1000))$
DD_0	degree-days below 0°C, chilling degree-days
DD5	degree-days above 5°C, growing degree-days
NFFD	the number of frost-free days
bFFP	the day of the year on which FFP begins
eFFP	the day of the year on which FFP ends
FFP	frost-free period
PAS	precipitation as snow (mm). For individual years, it covers the period between August in the previous year and July in the current year
EMT	extreme minimum temperature over 30 years
EXT	extreme maximum temperature over 30 years
Eref	Hargreaves reference evaporation (mm)
CMD	Hargreaves climatic moisture deficit (mm)
RH	mean annual relative humidity (%)

Table S4.4 The loadings of each climate variable of the first three principal components (PC) and the proportion of variance explained by each PC.

	Loadings		
	PC1	PC2	PC3
MAT	0.277	-0.003	0.014
MWMT	0.244	0.164	-0.052
MCMT	0.268	-0.085	0.030
TD	-0.195	0.253	-0.083
MAP	0.137	-0.422	0.155
MSP	-0.110	-0.395	0.201
AHM	-0.005	0.444	-0.060
SHM	0.175	0.297	-0.370
DD_0	-0.261	0.024	-0.055
DD5	0.272	0.037	-0.011
NFFD	0.269	-0.112	-0.053
bFFP	-0.247	0.120	0.144
eFFP	0.266	-0.094	-0.132
FFP	0.260	-0.108	-0.142
PAS	-0.246	-0.091	-0.051
EMT	0.267	-0.120	0.005
EXT	0.211	0.113	0.418
Eref	0.261	0.102	0.191
CMD	0.186	0.354	0.048
RH	0.000	-0.248	-0.710
Proportion of variance explained	0.651	0.193	0.071
Cumulative proportion of variance explained	0.651	0.844	0.915

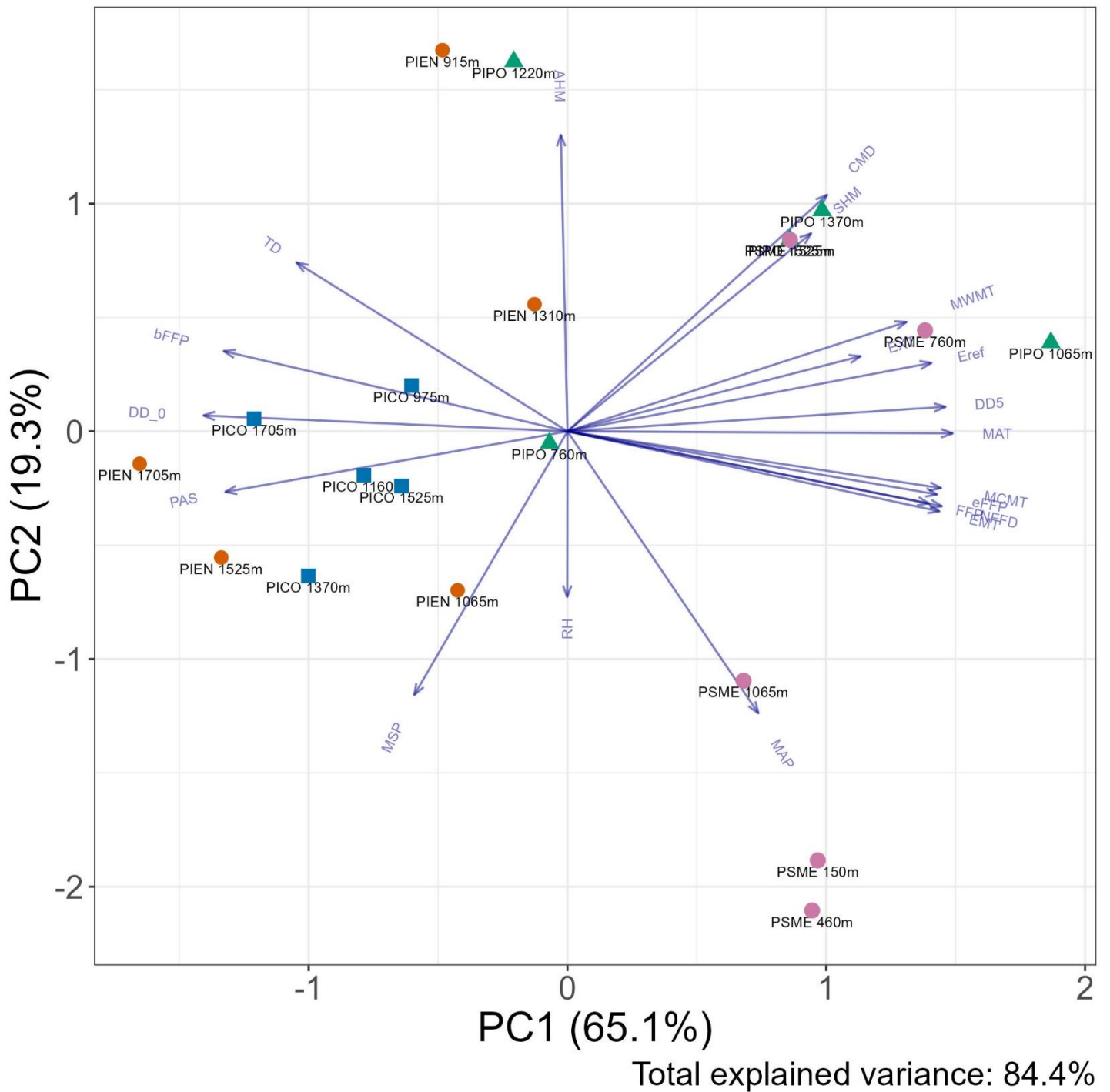


Figure S4.1. Principal Component Analysis (PCA) biplot showed that PC1 explains 65.1% of the variance and is related to mean annual temperature (MAT) and chilling (DD\_0) and growing degree days (DD5). PC2 explains 19.3% of the variance and is related to mean annual precipitation (MAP), mean May to September precipitation (MSP), and annual heat-moisture index. PIEN: *Picea engelmannii*; PICO: *Pinus contorta*; PIPO: *Pinus ponderosa*; PSME: *Pseudotsuga menziesii*.

Table S4.5. Seed germination percentage of *Pinus contorta* (PICO), *Picea engelmannii* (PIEN), *Pinus ponderosa* (PIPO), and *Pseudotsuga menziesii* (PSME) from different elevations at different temperatures showed significant differences but had no specific patterns at different elevations within the same temperature. Different letters (a, b, c, d) indicate significant differences between elevations within the same temperature (row-wise comparisons) by using Tukey's honest significance test (HSD). \*\*\*:  $p < 0.001$ ; \*\*:  $p < 0.01$ ; \*:  $p < 0.05$ .

Temperature (°C)	PICO Germination percentage (%)					
	975 m	1160 m	1370 m	1525 m	1705 m	<i>p</i> -value
5	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 1.8	0.3 ± 1.2	0.0 ± 0.0	0.056
10	0.3 ± 1.2	1.3 ± 2.6	2.3 ± 2.1	1.0 ± 1.8	0.7 ± 1.6	0.114
15	2.3 ± 3.2 <sup>bc</sup>	9.0 ± 6.2 <sup>b</sup>	20.0 ± 9.2 <sup>a</sup>	7.7 ± 7.9 <sup>bc</sup>	1.7 ± 2.7 <sup>c</sup>	<0.001***
20	70.7 ± 15.3	62.0 ± 11.9	74.3 ± 16.8	63.7 ± 15.3	72.3 ± 15.6	0.195
25	82.0 ± 9.3 <sup>a</sup>	53.7 ± 18.0 <sup>c</sup>	80.0 ± 10.7 <sup>a</sup>	63.7 ± 10.8 <sup>bc</sup>	73.0 ± 8.2 <sup>ab</sup>	<0.001***
30	57.7 ± 9.4 <sup>a</sup>	23.7 ± 7.5 <sup>d</sup>	47.7 ± 13.4 <sup>ab</sup>	28.0 ± 9.8 <sup>cd</sup>	36.0 ± 10.4 <sup>bc</sup>	<0.001***
35	27.3 ± 11.5 <sup>a</sup>	11.0 ± 8.5 <sup>b</sup>	17.3 ± 9.7 <sup>ab</sup>	28.0 ± 14.4 <sup>a</sup>	20.0 ± 8.5 <sup>ab</sup>	<0.01**
40	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	–
Temperature (°C)	PIEN Germination percentage (%)					
	915 m	1065 m	1310 m	1525 m	1705 m	<i>p</i> -value
5	14.7 ± 7.1 <sup>a</sup>	1.3 ± 2.0 <sup>b</sup>	4.7 ± 4.1 <sup>b</sup>	2.3 ± 4.0 <sup>b</sup>	0.3 ± 1.2 <sup>b</sup>	<0.001***
10	14.3 ± 4.3 <sup>a</sup>	2.0 ± 3.2 <sup>b</sup>	10.0 ± 7.5 <sup>a</sup>	11.7 ± 5.8 <sup>a</sup>	2.7 ± 3.9 <sup>b</sup>	<0.001***
15	72.9 ± 7.5 <sup>a</sup>	58.3 ± 9.3 <sup>b</sup>	71.0 ± 10.5 <sup>a</sup>	40.7 ± 10.9 <sup>c</sup>	67.0 ± 11.2 <sup>ab</sup>	<0.001***
20	86.3 ± 5.5 <sup>b</sup>	98.0 ± 4.0 <sup>a</sup>	94.3 ± 5.8 <sup>a</sup>	69.7 ± 10.0 <sup>c</sup>	92.7 ± 5.1 <sup>ab</sup>	<0.001***
25	83.3 ± 12.5 <sup>a</sup>	87.0 ± 5.9 <sup>a</sup>	82.3 ± 11.4 <sup>a</sup>	59.0 ± 14.9 <sup>b</sup>	87.0 ± 7.5 <sup>a</sup>	<0.001***
30	68.0 ± 10.7 <sup>a</sup>	61.7 ± 8.9 <sup>a</sup>	70.7 ± 10.4 <sup>a</sup>	36.3 ± 12.4 <sup>b</sup>	63.3 ± 12.4 <sup>a</sup>	<0.001***
35	11.7 ± 17.4 <sup>ab</sup>	26.0 ± 19.6 <sup>a</sup>	4.0 ± 5.9 <sup>b</sup>	3.3 ± 8.0 <sup>b</sup>	6.7 ± 11.0 <sup>b</sup>	<0.001***
40	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	–
Temperature (°C)	PIPO Germination percentage (%)					
	760 m	1065 m	1220 m	1370 m	1525 m	<i>p</i> -value
5	3.3 ± 4.8 <sup>b</sup>	0.0 ± 0.0 <sup>b</sup>	3.0 ± 3.9 <sup>b</sup>	3.0 ± 3.5 <sup>b</sup>	10.7 ± 9.2 <sup>a</sup>	<0.001***
10	6.0 ± 4.7 <sup>b</sup>	0.7 ± 1.6 <sup>b</sup>	4.7 ± 5.1 <sup>b</sup>	6.7 ± 4.6 <sup>b</sup>	19.0 ± 10.5 <sup>a</sup>	<0.001***
15	4.8 ± 3.6 <sup>bc</sup>	2.0 ± 2.7 <sup>c</sup>	11.3 ± 7.2 <sup>b</sup>	8.7 ± 5.1 <sup>bc</sup>	24.3 ± 11.4 <sup>a</sup>	<0.001***

20	41.0 ± 10.0 <sup>ab</sup>	15.7 ± 11.5 <sup>c</sup>	50.3 ± 17.6 <sup>a</sup>	29.3 ± 9.1 <sup>bc</sup>	44.0 ± 15.2 <sup>ab</sup>	<0.001***
25	43.7 ± 12.8 <sup>ab</sup>	14.7 ± 7.3 <sup>c</sup>	53.0 ± 11.5 <sup>a</sup>	37.0 ± 12.8 <sup>b</sup>	34.4 ± 9.9 <sup>b</sup>	<0.001***
30	45.0 ± 13.0 <sup>a</sup>	16.3 ± 10.0 <sup>c</sup>	41.0 ± 16.6 <sup>ab</sup>	29.7 ± 8.4 <sup>bc</sup>	29.7 ± 7.5 <sup>bc</sup>	<0.001***
35	10.7 ± 13.8	2.0 ± 4.0	8.0 ± 8.9	15.0 ± 15.2	12.7 ± 9.8	0.056
40	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	–
Temperature (°C)	PSME Germination percentage (%)					<i>p</i> -value
	150 m	460 m	760 m	1065 m	1525 m	
5	0.0 ± 0.0 <sup>a</sup>	0.0 ± 0.0 <sup>a</sup>	1.3 ± 2.0 <sup>a</sup>	0.7 ± 1.6 <sup>a</sup>	1.7 ± 2.1 <sup>a</sup>	<0.05*
10	3.3 ± 4.8 <sup>ab</sup>	1.3 ± 2.0 <sup>b</sup>	8.0 ± 4.8 <sup>a</sup>	4.7 ± 3.7 <sup>ab</sup>	6.3 ± 4.7 <sup>a</sup>	<0.01**
15	8.1 ± 8.1 <sup>b</sup>	3.0 ± 3.5 <sup>b</sup>	17.0 ± 7.8 <sup>a</sup>	5.7 ± 4.7 <sup>b</sup>	5.7 ± 4.7 <sup>b</sup>	<0.001***
20	52.7 ± 11.7	51.0 ± 20.5	42.7 ± 17.8	44.7 ± 11.4	39.7 ± 12.8	0.194
25	75 ± 8.4 <sup>a</sup>	74.3 ± 8.6 <sup>a</sup>	44.7 ± 14.2 <sup>b</sup>	53.0 ± 25.9 <sup>b</sup>	51.3 ± 13.7 <sup>b</sup>	<0.001***
30	55 ± 10.4 <sup>a</sup>	60.0 ± 9.0 <sup>a</sup>	36.0 ± 12.9 <sup>b</sup>	40.3 ± 16.4 <sup>b</sup>	31.3 ± 9.8 <sup>b</sup>	<0.001***
35	1.7 ± 2.7	3.7 ± 4.0	3.3 ± 5.1	3.7 ± 4.0	1.7 ± 3.6	0.504
40	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	–

Table S4.6. The optimal temperature for seed germination ( $T_o$ ), shape coefficient ( $b$ ), and germination portion at optimal temperature ( $G_{max}$ ) of *Pinus contorta* (PICO), *Picea engelmannii* (PIEN), *Pinus ponderosa* (PIPO), and *Pseudotsuga menziesii* (PSME) from different elevations showed differences in some traits. Different letters (a, b, c) indicate significant differences between elevations within the same species (row-wise comparisons) by using Tukey's honest significance test (HSD). \*\*\*:  $p < 0.001$ ; \*\*:  $p < 0.01$ ; \*:  $p < 0.05$ .

Species	Optimal germination temperature ( $T_o$ , °C)					
PICO	975 m	1160 m	1370 m	1525 m	1705 m	$p$ -value
	25.1 ± 0.5 <sup>a</sup>	22.8 ± 0.3 <sup>b</sup>	23.8 ± 0.3 <sup>ab</sup>	23.8 ± 1.1 <sup>ab</sup>	23.7 ± 0.5 <sup>ab</sup>	<0.05*
PIEN	915 m	1065 m	1310 m	1525 m	1705 m	
	22.1 ± 0.9	22.9 ± 0.7	22.1 ± 0.5	21.8 ± 0.9	22.3 ± 0.9	0.544
PIPO	760 m	1065 m	1220 m	1370 m	1525 m	
	25.6 ± 0.9 <sup>a</sup>	25.5 ± 3.0 <sup>a</sup>	24.3 ± 0.9 <sup>a</sup>	25.3 ± 0.4 <sup>a</sup>	21.5 ± 0.9 <sup>b</sup>	<0.05*
PSME	150 m	460 m	760 m	1065 m	1525 m	
	25.0 ± 0.3	25.4 ± 0.5	23.6 ± 1.1	24.8 ± 0.6	24.3 ± 0.7	0.069
	Shape coefficient ( $b$ )					
PICO	975 m	1160 m	1370 m	1525 m	1705 m	
	5.6 ± 0.1 <sup>a</sup>	4.7 ± 0.4 <sup>b</sup>	5.7 ± 0.2 <sup>a</sup>	5.4 ± 0.6 <sup>a</sup>	4.8 ± 0.2 <sup>b</sup>	<0.05*
PIEN	915 m	1065 m	1310 m	1525 m	1705 m	
	7.5 ± 0.6	7.0 ± 0.4	7.2 ± 0.4	6.4 ± 0.1	6.8 ± 0.6	0.084
PIPO	760 m	1065 m	1220 m	1370 m	1525 m	
	6.0 ± 0.3 <sup>b</sup>	4.5 ± 1.8 <sup>b</sup>	5.7 ± 0.3 <sup>b</sup>	6.8 ± 0.2 <sup>ab</sup>	9.0 ± 0.9 <sup>a</sup>	<0.01**
PSME	150 m	460 m	760 m	1065 m	1525 m	
	5.0 ± 0.2	4.9 ± 0.4	6.3 ± 0.6	5.3 ± 0.9	4.9 ± 0.5	0.066
	Germination portion at optimal temperature ( $G_{max}$ )					
PICO	975 m	1160 m	1370 m	1525 m	1705 m	
	0.88 ± 0.03	0.66 ± 0.12	0.86 ± 0.11	0.70 ± 0.13	0.83 ± 0.06	0.078
PIEN	915 m	1065 m	1310 m	1525 m	1705 m	
	0.96 ± 0.02 <sup>a</sup>	0.98 ± 0.02 <sup>a</sup>	0.95 ± 0.04 <sup>a</sup>	0.71 ± 0.04 <sup>b</sup>	0.95 ± 0.06 <sup>a</sup>	<0.001***
PIPO	760 m	1065 m	1220 m	1370 m	1525 m	
	0.52 ± 0.08 <sup>ab</sup>	0.23 ± 0.06 <sup>c</sup>	0.59 ± 0.03 <sup>a</sup>	0.38 ± 0.06 <sup>bc</sup>	0.40 ± 0.08 <sup>bc</sup>	<0.001***
PSME	150 m	460 m	760 m	1065 m	1525 m	
	0.80 ± 0.02	0.82 ± 0.08	0.50 ± 0.15	0.59 ± 0.16	0.55 ± 0.12	<0.05*

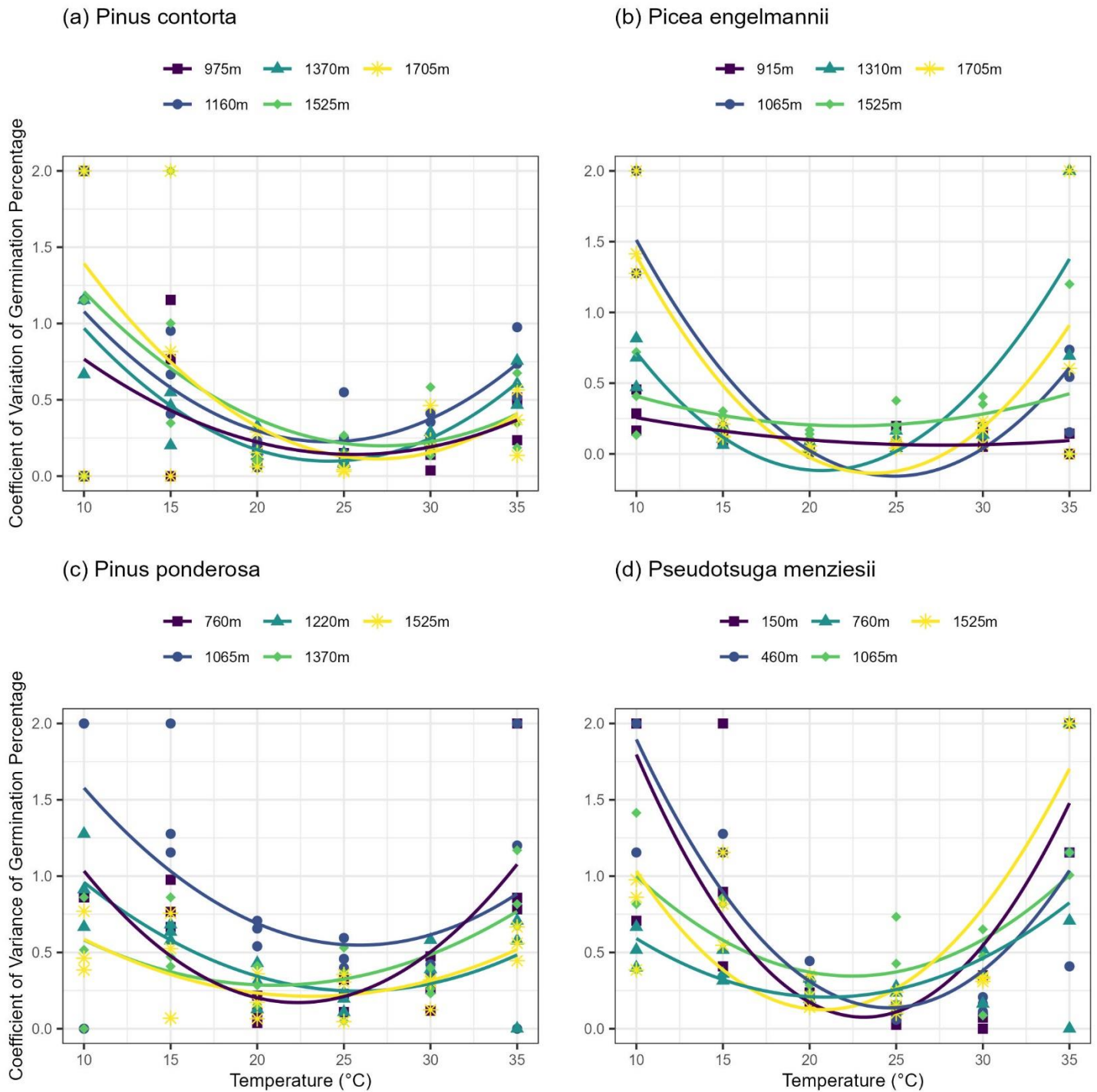


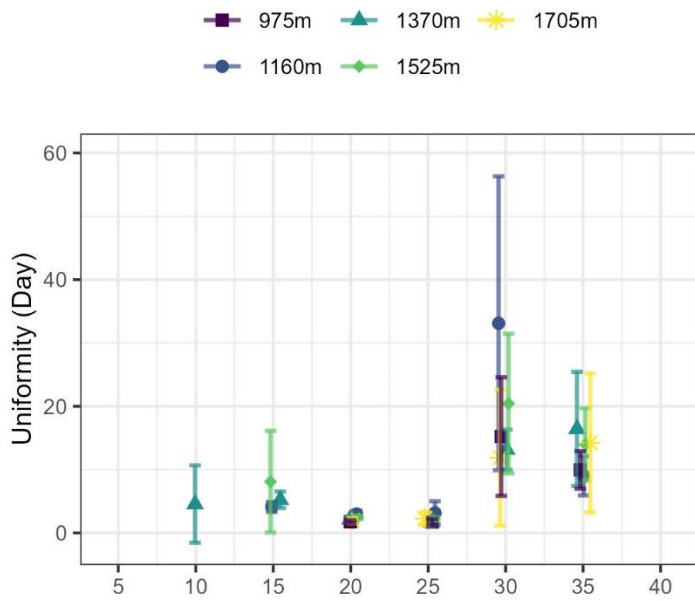
Figure S4.2. The coefficient of variance of germination percentage in *Pinus contorta* (PICO), *Picea engelmannii* (PIEN), *Pinus ponderosa* (PIPO), and *Pseudotsuga menziesii* (PSME) from different elevations at different temperatures showed variations are smaller near the optimal temperature and are larger when temperatures are suboptimal to near the limited temperatures.

Table S4.7. The optimal temperature ( $T_o$ ), shape coefficient ( $b$ ), and  $GR_{50}$  at the optimal temperature ( $G_{max}$ ) for  $GR_{50}$  of *Pinus contorta* (PICO), *Picea engelmannii* (PIEN), *Pinus ponderosa* (PIPO), and *Pseudotsuga menziesii* (PSME) from different elevations. Different letters (a and b) indicate significant differences between elevations within the same species (row-wise comparisons) by using Tukey's honest significance test (HSD).

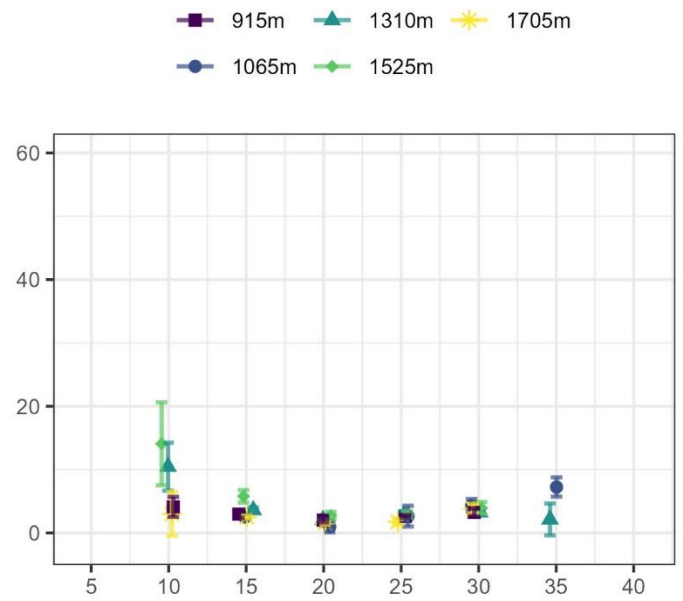
\*\*\*:  $p < 0.001$ ; \*\*:  $p < 0.01$ ; \*:  $p < 0.05$ .

Species	$GR_{50}$ Optimal temperature ( $T_o$ , °C)					
PICO	975 m	1160 m	1370 m	1525 m	1705 m	$p$ -value
	24.1 ± 1.2	23.8 ± 1.0	24.2 ± 0.7	23.4 ± 1.4	25.1 ± 1.4	0.541
PIEN	915 m	1065 m	1310 m	1525 m	1705 m	$p$ -value
	22.0 ± 0.7 <sup>b</sup>	23.3 ± 0.9 <sup>ab</sup>	23.6 ± 0.5 <sup>a</sup>	23.0 ± 0.1 <sup>ab</sup>	22.8 ± 0.3 <sup>ab</sup>	0.051
PIPO	760 m	1065 m	1220 m	1370 m	1525 m	$p$ -value
	24.7 ± 2.9	26.0 ± 4.1	24.4 ± 5.4	24.7 ± 3.8	26.4 ± 2.4	0.949
PSME	150 m	460 m	760 m	1065 m	1525 m	$p$ -value
	21.6 ± 1.3 <sup>ab</sup>	20.4 ± 1.2 <sup>b</sup>	22.5 ± 1.3 <sup>ab</sup>	24.3 ± 0.5 <sup>a</sup>	23.1 ± 1.8 <sup>ab</sup>	<0.05*
$GR_{50}$ shape coefficient ( $b$ )						
PICO	975 m	1160 m	1370 m	1525 m	1705 m	$p$ -value
	5.5 ± 1.1	6.8 ± 1.7	6.4 ± 0.9	6.2 ± 0.3	5.8 ± 1.1	0.639
PIEN	915 m	1065 m	1310 m	1525 m	1705 m	$p$ -value
	7.5 ± 0.5	6.9 ± 0.7	7.9 ± 0.5	6.7 ± 0.4	7.1 ± 0.9	0.243
PIPO	760 m	1065 m	1220 m	1370 m	1525 m	$p$ -value
	11.8 ± 1.8 <sup>a</sup>	6.1 ± 0.1 <sup>b</sup>	9.4 ± 1.8 <sup>ab</sup>	11.4 ± 3.4 <sup>a</sup>	9.7 ± 1.1 <sup>ab</sup>	<0.05*
PSME	150 m	460 m	760 m	1065 m	1525 m	$p$ -value
	9.8 ± 1.2	9.6 ± 1.0	8.9 ± 0.7	9.8 ± 1.6	10.6 ± 1.4	0.621
$GR_{50}$ at optimal temperature ( $G_{max}$ )						
PICO	975 m	1160 m	1370 m	1525 m	1705 m	$p$ -value
	0.26 ± 0.04	0.18 ± 0.03	0.22 ± 0.03	0.21 ± 0.01	0.24 ± 0.03	0.125
PIEN	915 m	1065 m	1310 m	1525 m	1705 m	$p$ -value
	0.22 ± 0.01	0.24 ± 0.04	0.20 ± 0.02	0.20 ± 0.0	0.25 ± 0.01	0.054
PIPO	760 m	1065 m	1220 m	1370 m	1525 m	$p$ -value
	0.06 ± 0.01	0.10 ± 0.03	0.10 ± 0.06	0.09 ± 0.04	0.10 ± 0.01	0.548
PSME	150 m	460 m	760 m	1065 m	1525 m	$p$ -value
	0.09 ± 0.01 <sup>b</sup>	0.09 ± 0.01 <sup>b</sup>	0.12 ± 0.01 <sup>a</sup>	0.08 ± 0.01 <sup>b</sup>	0.08 ± 0.01 <sup>b</sup>	<0.001***

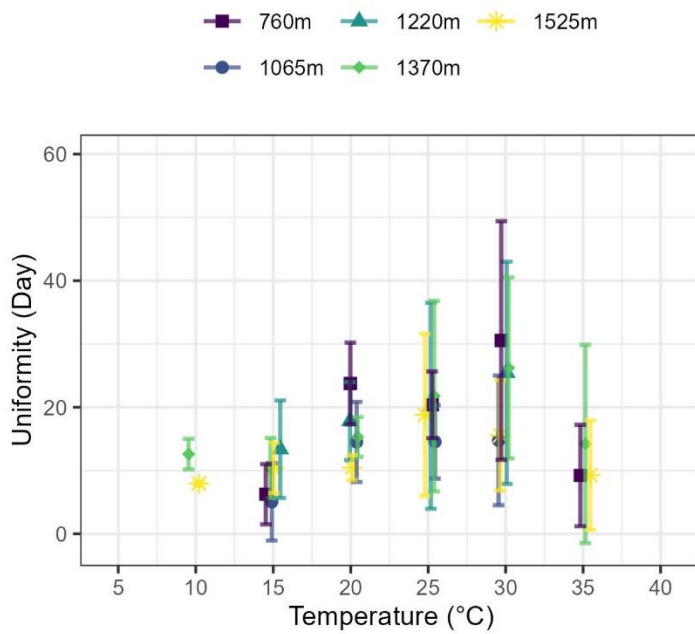
(a) *Pinus contorta*



(b) *Picea engelmannii*



(c) *Pinus ponderosa*



(d) *Pseudotsuga menziesii*

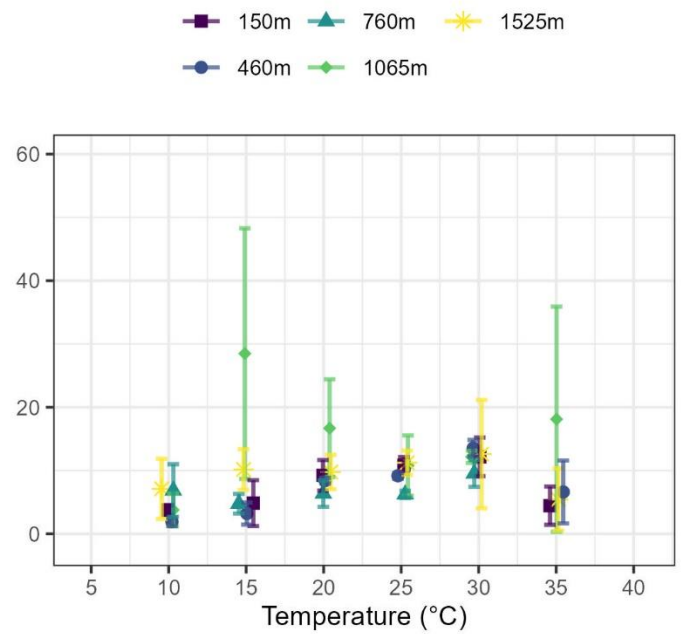


Figure S4.3. The uniformity of (a) *Picea engelmannii* and (b) *Pinus contorta* in all experimental rounds at different temperatures showed that the uniformity decreased when the temperatures shifted from optimum toward upper or lower limits. However, there was no specific pattern in (c) *Pinus ponderosa* and (d) *Pseudotsuga menziesii*. Points and error bars were jittered horizontally for better visualization.

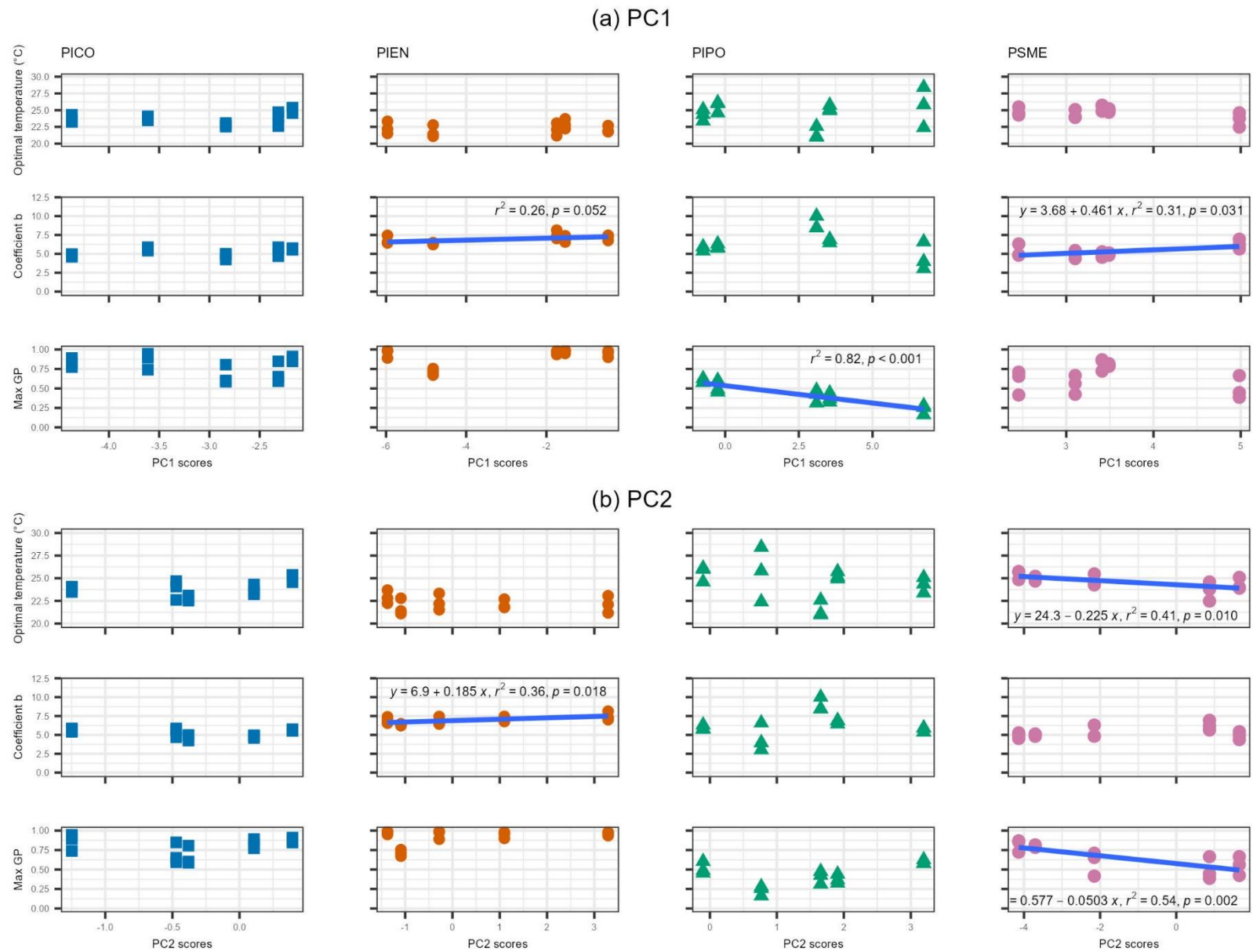


Figure S4.4. Intra-species relationships between PC1 (a) and PC2 (b) and seed germination traits of *Picea engelmannii* (PIEN); *Pinus contorta* (PICO); *Pinus ponderosa* (PIPO); *Pseudotsuga menziesii* (PSME).

Table S4.8. The slopes of significant trends in each species and temperature along PC1 and PC2. *Pinus contorta* (PICO), *Picea engelmannii* (PIEN), *Pinus ponderosa* (PIPO), and *Pseudotsuga menziesii* (PSME). Negative (blue font) and positive (red font) slopes mean germination percentages decrease and increase, respectively, with increasing PC1 or PC2 scores. –: no significant trend; \*\*\*:  $p < 0.001$ ; \*\*:  $p < 0.01$ ; \*:  $p < 0.05$ . Lower PC1 scores are colder sites, and higher PC1 scores are warmer sites. Lower PC2 scores are warmer and wetter sites, and higher PC2 scores are colder and wet but relatively drier than sites with higher PC2 scores. †Optimal is the optimal temperature derived from the Gaussian function in each species (Table 4.3).

Temperature (°C)	Species							
	PICO		PIEN		PIPO		PSME	
	PC1							
	Slope	<i>p</i> -value	Slope	<i>p</i> -value	Slope	<i>p</i> -value	Slope	<i>p</i> -value
5	–	–	–	–	–	–	–	–
10	–	–	–	–	–	–	–	–
15	–	–	–	–	–	–	0.050	<0.01**
20	–	–	–	–	-0.040	<0.01**	–	–
Optimal †	23.8°C		22.2°C		24.4°C		24.6°C	
25	–	–	–	–	-0.045	<0.001***	–	–
30	–	–	–	–	-0.036	<0.001***	–	–
35	–	–	–	–	–	–	–	–
40	–	–	–	–	–	–	–	–
Temperature (°C)	PC2							
	Slope	<i>p</i> -value	Slope	<i>p</i> -value	Slope	<i>p</i> -value	Slope	<i>p</i> -value
5	-0.006	<0.05*	0.028	<0.001***	–	–	0.003	<0.05*
10	-0.012	<0.01**	0.020	<0.05*	–	–	0.009	<0.01**
15	-0.113	<0.001***	0.050	<0.01**	–	–	–	–
20	–	–	–	–	–	–	–	–
Optimal †	23.8°C		22.2°C		24.4°C		24.6°C	
25	–	–	–	–	–	–	-0.047	<0.01**
30	–	–	0.041	<0.05*	–	–	-0.044	<0.001***
35	–	–	–	–	–	–	–	–
40	–	–	–	–	–	–	–	–

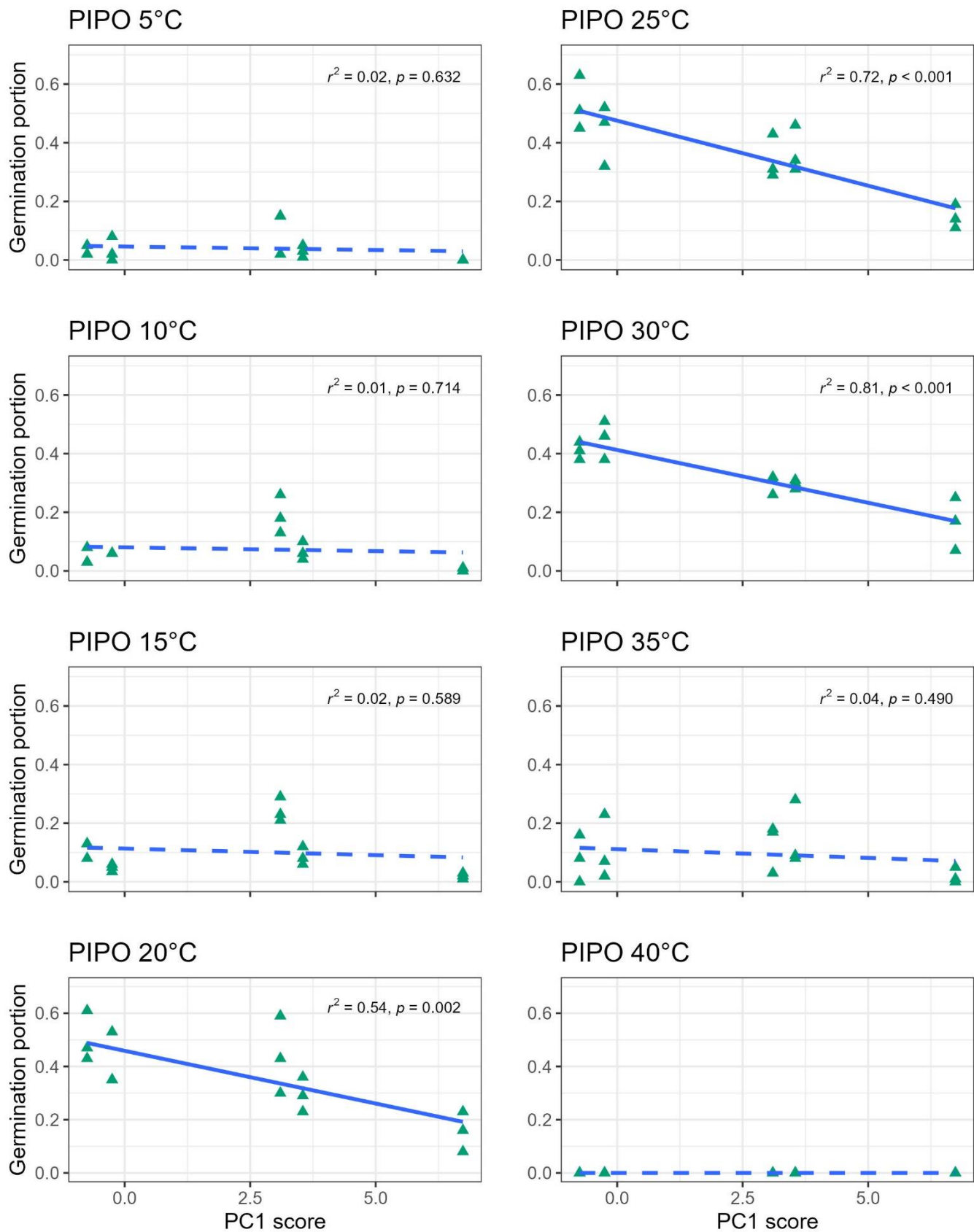


Figure S4.5. Intra-species relationships between PC1 and germination portions of *Pinus ponderosa* (PIPO) at different temperatures. Dashed line: non-significant trend. Solid lines: significant trends.

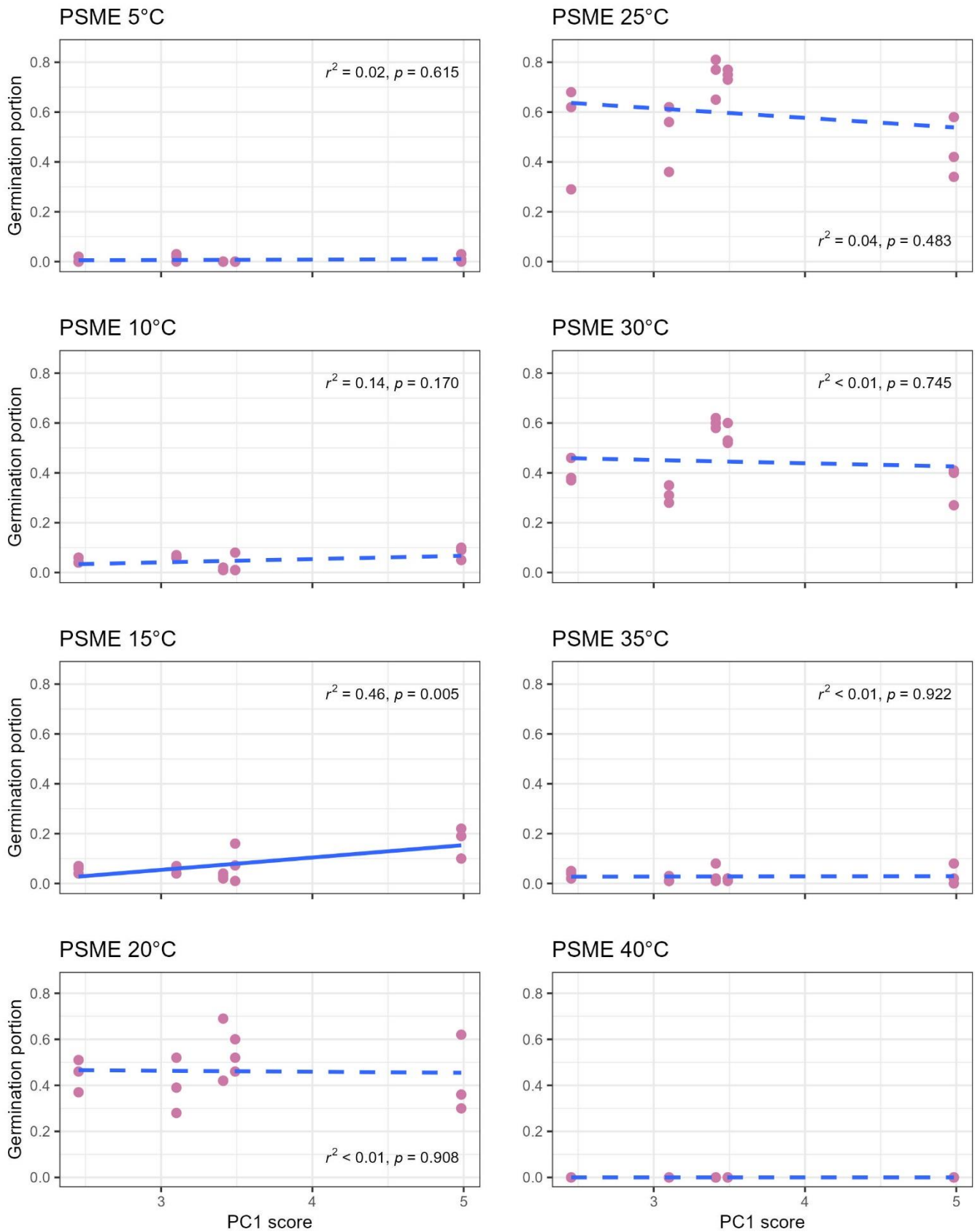


Figure S4.6. Intra-species relationships between PC1 and germination portions of *Pseudotsuga menziesii* (PSME) at different temperatures. Dashed line: non-significant trend. Solid lines: significant trends.

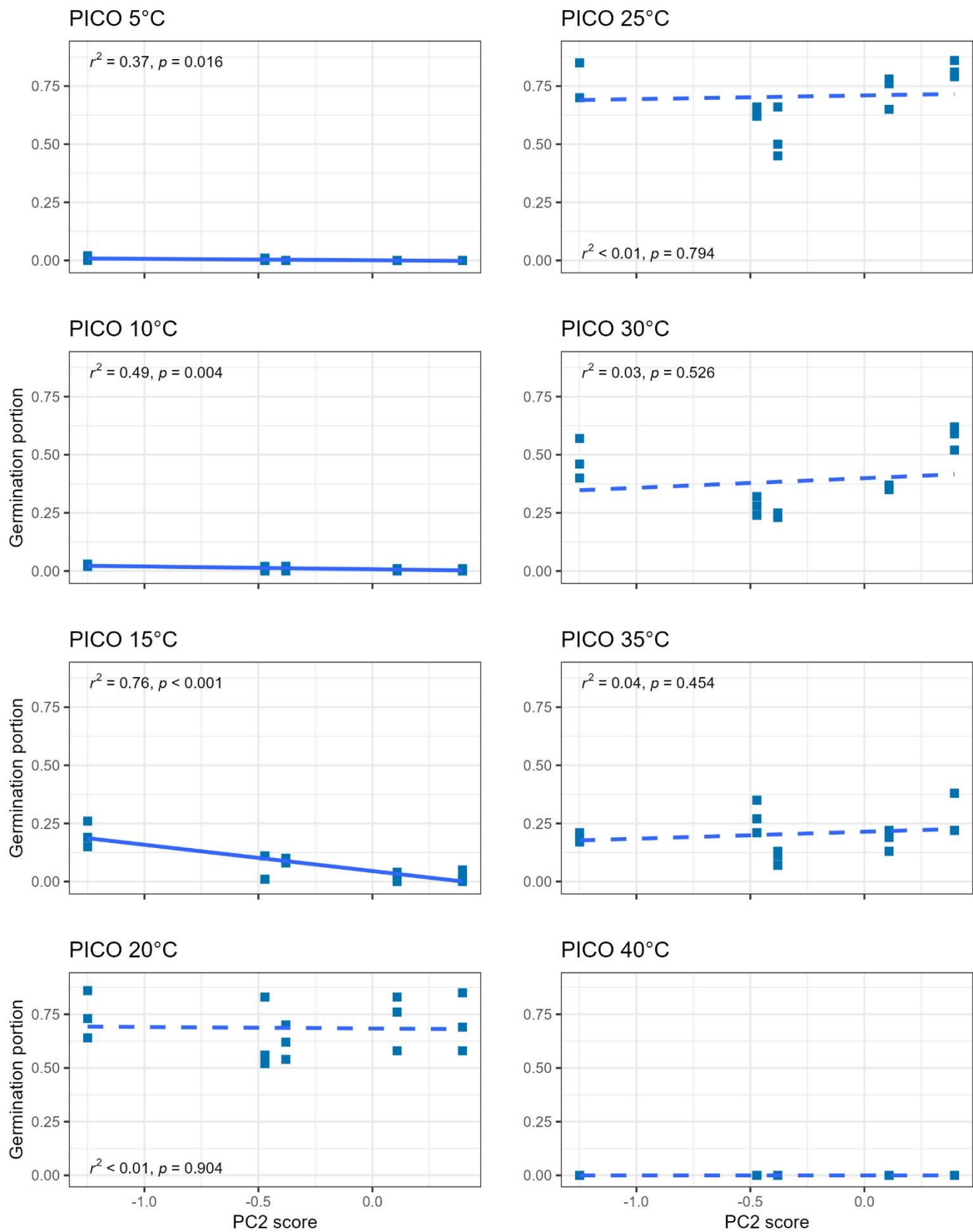


Figure S4.7. Relationships between PC2 and germination portions of *Pinus contorta* (PICO) at different temperatures. Dashed line: non-significant trend. Solid lines: significant trends.

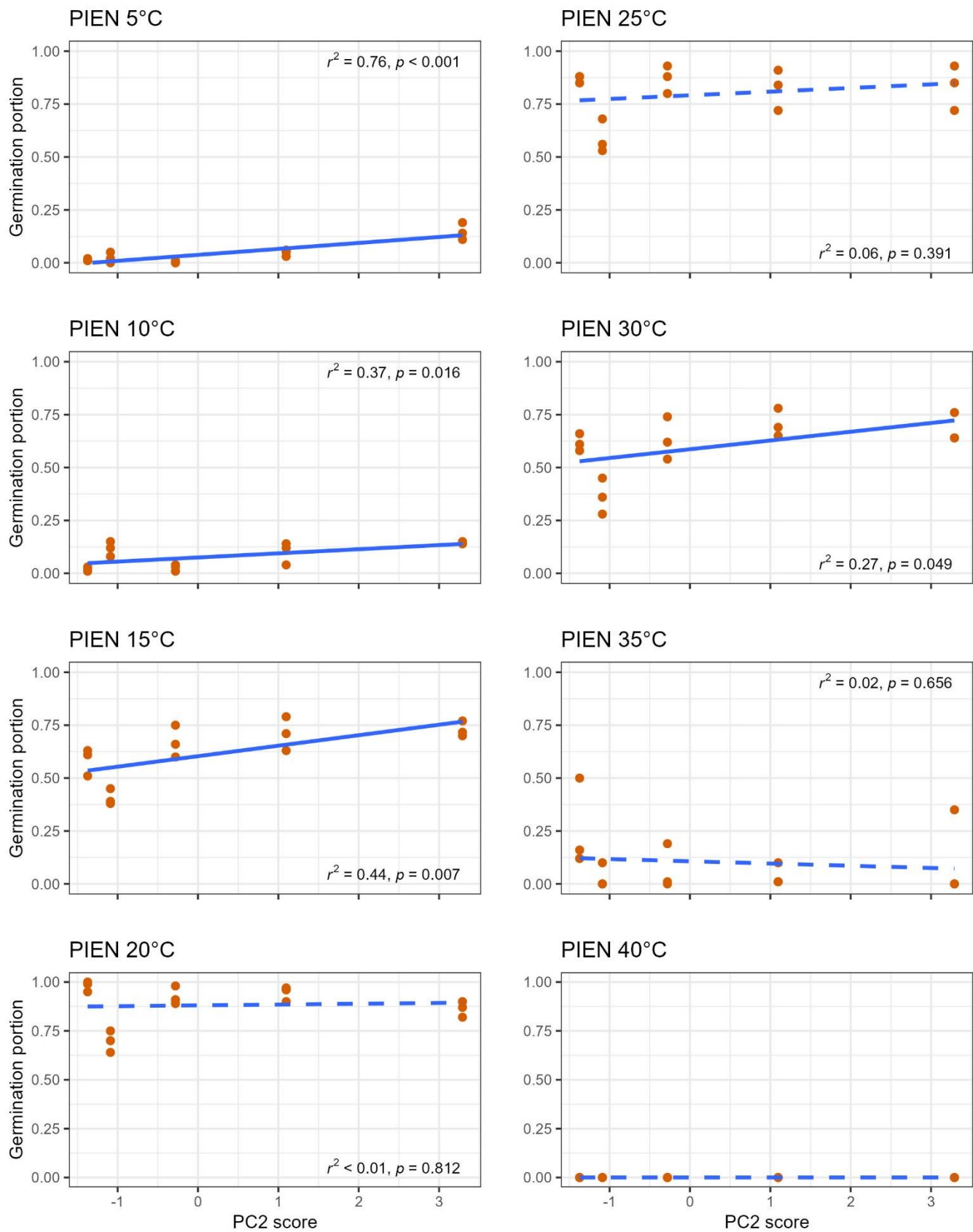


Figure S4.8. Relationships between PC2 and germination portions of *Picea engelmannii* (PIEN) at different temperatures. Dashed line: non-significant trend. Solid lines: significant trends.

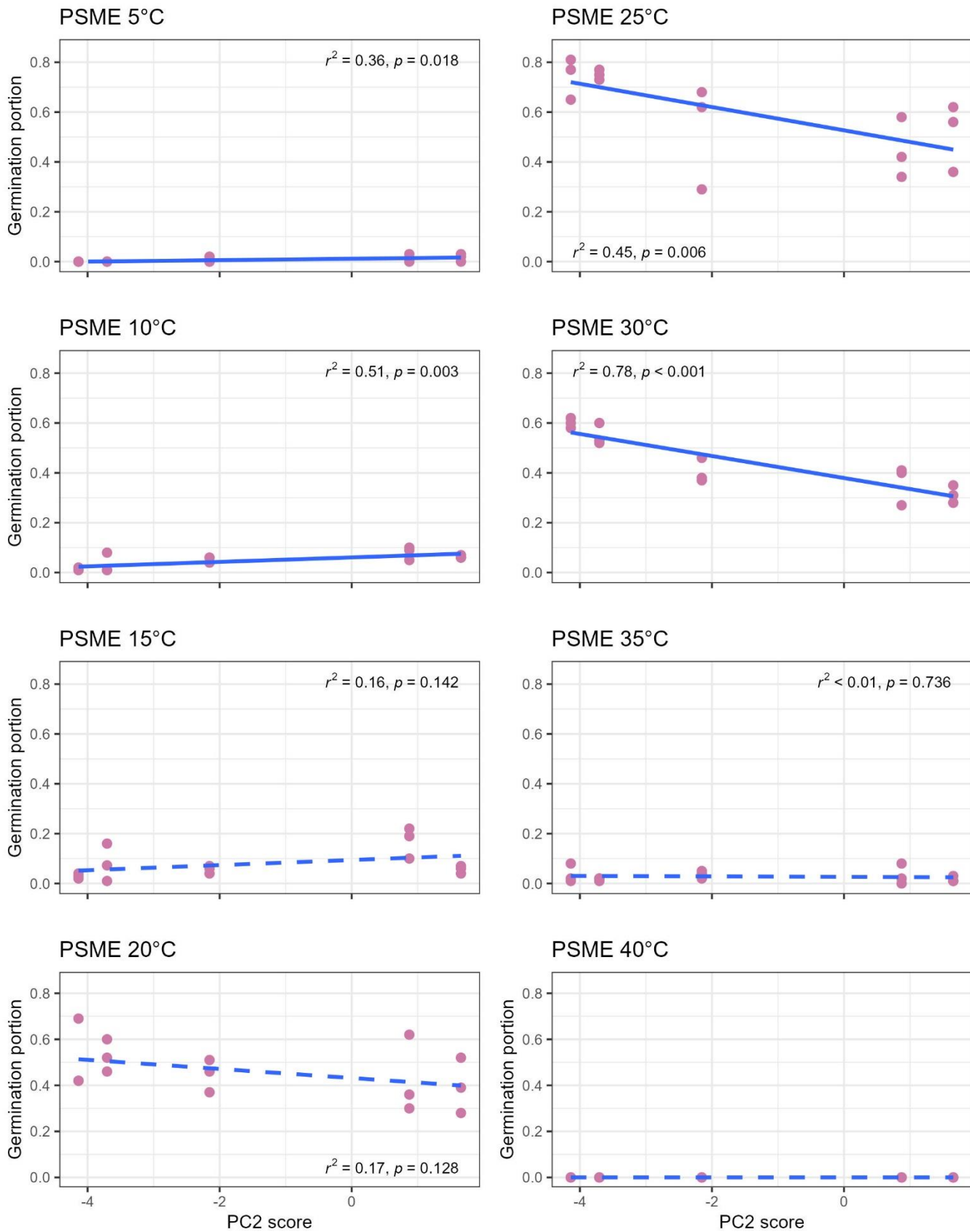


Figure S4.9. Relationships between PC2 and germination portions of *Pseudotsuga menziesii* (PSME) at different temperatures. Dashed line: non-significant trend. Solid lines: significant trends.

Table S4.9. ANCOVA results of the optimal temperature ( $T_o$ ), shape coefficient ( $b$ ), and germination portion at optimal temperature ( $G_{max}$ ). It showed seed ages had no effect on the optimal temperature and shape coefficient  $b$  but significantly affected the germination portion at the optimal temperature. \*\*\*:  $p < 0.001$ ; \*\*:  $p < 0.01$ ; \*:  $p < 0.05$ .

	p-value		
	Optimal temperature ( $T_o$ )	Shape coefficient ( $b$ )	Germination portion at optimal temperature ( $G_{max}$ )
Species	<0.01**	<0.05*	<0.001***
Elevations	0.165	0.17	<0.001***
Seed ages	0.307	0.44	<0.001***

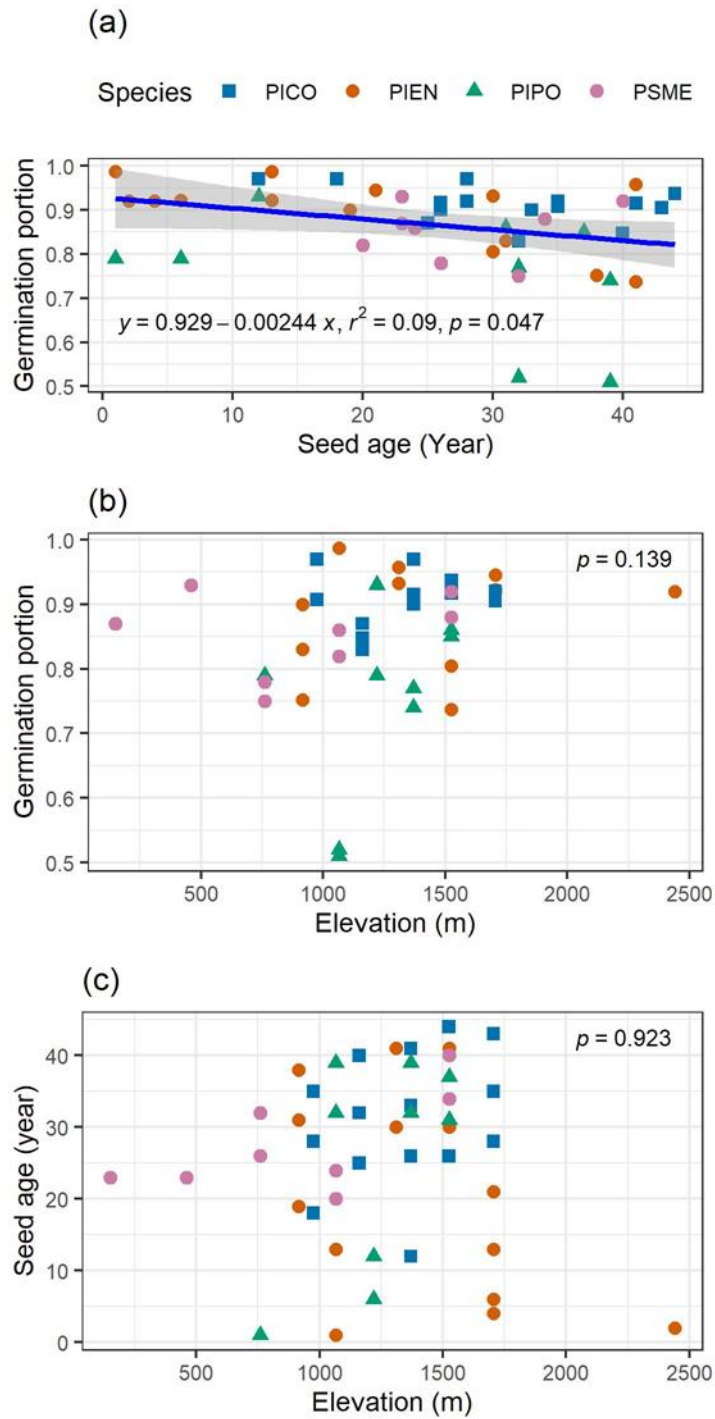


Figure S4.10. The seed germination portion decreased with increasing seed ages (a). There was no relationship between seed germination portions and elevations (b) and elevations and seed ages (c).

## Chapter 5

### Conclusions and Future Directions

#### 5.1 Conclusions

The goals of this study are to (1) evaluate the effects of winter warming versus spring warming on flowering phenology and how these warming in different periods assert variable effects on flowering dates, (2) explore how temperature dependence of pollen germination and tube growth varies among different conifer species and populations of the same species collected from various elevations, and (3) examine how temperature dependence of seed germination varies among different conifer species and populations of the same species collected from various elevations and whether the variation of germination traits relates to any climate factors at seed-collecting sites. In this study, I demonstrated that temperatures significantly affect reproduction processes and reproductive phases, e.g., flowering phenology, pollen germination, pollen tube growth, and seed germination are the most temperature-vulnerable stages. Therefore, the impact of climate change on reproduction could be a major limitation on tree distribution. Here, I summarize the main conclusions in each chapter. The results can contribute to more effective climate adaptation strategies, e.g., seed transfer, assisted migration, locating corridors and refugia, etc., for policymakers and stakeholders, help mitigate climate change impact, and increase our understanding of forest resilience.

5.1.1 Rates of change in bloom dates differ significantly between before and after 1980, and excessive warming could delay blooms, which has manifested at low latitudes.

This study illustrates that warming during chilling and forcing periods shifted bloom dates of Yoshino cherries differentially and exerted varying effects on bloom time across different latitudes in Japan. Forcing temperatures consistently advanced bloom dates irrespective of chilling temperatures at all locations. In contrast, the chilling temperatures significantly affected the bloom dates at higher and lower latitude locations in different directions. That is, advancing

at high latitudes and delaying at low latitudes. These findings support the slowdown of phenological shifts caused by warmer winters in recent years. The temperature sensitivities of chilling and forcing periods in this study provide quantitative evidence showing that these counteracting effects in spring phenology due to warming during the chilling period are observed in recent records from low-latitude locations in Japan. These findings highlight that the flowering time could be delayed rather than advanced under temperature warming. This phenomenon has begun to emerge in low-latitude locations and may extend to higher latitudes in the future.

5.1.2 Optimal temperature and temperature ranges for pollen germination and tube growth vary among species and elevations within the same species and reflect the acclimation or adaptation of species to spring mean temperatures.

Pollen germination and tube growth are highly sensitive to climatic conditions and may vary among species and populations. The results illustrate the temperature dependence of pollen germination and tube growth across a wide range of temperatures of three conifer species from the elevational gradient. They show that pollen from different species and different elevations within the same species had different germination percentages, tube lengths, and optimal temperatures that are correlated with their ecological niche. In addition, the population with a higher optimal temperature for germination will also have a higher optimal temperature for pollen tube growth, and the tube growth optimal temperature is higher than the optimal temperature for germination. Another finding is that the variations of pollen germination are larger when the temperature approaches the upper and lower biological limits of germination. Conversely, the variations of tube growth are larger when the temperature is optimal. The impacts of climate change on reproduction could be a major limitation on temperate tree distribution. Determining the temperature dependence of conifer pollen germination and pollen tube growth from different conifer species and populations is essential to evaluating species' response to temperature for predicting and mitigating the impacts of climate change on plant populations and ecosystems. This study in temperature requirements of pollen germination and tube growth in vitro could be integrated into species

distribution models as they can quantitatively estimate reproduction success under specific climatic conditions.

5.1.3 Seeds from different species and different elevations within the same species had different germination traits that are correlated with their ecological niche, and germination traits do not show the environmental gradients.

Seed germination is highly sensitive to climatic conditions and may vary among species and populations. The results illustrate the temperature dependence of seed germination across a wide range of temperatures of four conifer species from different locations and elevations. They show that seeds from different species and different elevations within the same species had different germination traits correlated with their ecological niche. However, the germination traits do not show the environmental gradients we expected. We also found that the variations of germination traits are larger when the temperature approaches the upper and lower biological limits of germination. Although we cannot predict this intraspecific variation of seed germination traits, the variation may increase species resilience, improve the chance of species persistence under climate change, and mitigate species' vulnerability to changing climate. Seed germination traits have been neglected in vegetation science compared with seed morphological traits. Determining the temperature dependence of seed germination from different provenances is essential to evaluate species' response to temperature for predicting and mitigating the impacts of climate change on plant populations and ecosystems. We anticipate applying these understandings to predict population dynamics and range shifts under climate warming and expect this information can also help with adjusting forest management practices, seed transfer, and assisted migration.

## 5.2 Future directions

This study has illustrated interesting points of temperature dependences of tree reproductive processes, including flowering phenology, pollen viability, and seed germination. Still, it requires improvements and attention to many knowledge gaps that limit a complete

understanding of how climate change influences conifer reproduction and distribution. Based on the findings from this study, I discuss study limitations, applications, and future directions to improve our knowledge in conifer reproduction responses and distribution to climate change.

### 5.2.1 Phenological study of conifer male and female cones

Long-term phenological data on reproductive biology in trees are rare, especially in conifers. Without long-term conifer flowering phenology data, we cannot do the same analysis as we did for the Yoshino cherry. In order to investigate the warming effects on male and female cones to see whether warming will create a mismatch in conifer reproduction, increasing flowering phenological observations of conifer species in the future is strongly recommended. Unlike the Yoshino cherry, where warming may create a phenological mismatch between trees and insects, conifer pollination is facilitated by wind. The study of conifer flowering phenology should focus on investigating the mismatch between the timing of male and female cones that warming creates. That is, the synchronicity between pollen shedding and ovules receptive. This mismatch could further affect conifer reproduction, including the production and quality of seeds and, thus, their regeneration potential. Although we can apply the same method to conifer reproduction, we must acknowledge the limitations of the Partial Least Squares (PLS) regression. We define chilling and forcing periods by using PLS regression (Luedeling & Gassner, 2012; Wenden et al., 2017) to analyze the influence of daily temperatures on cherry bloom dates. However, the criteria to define the start and end days are subjective unless a method similar to the approach used by Wenden et al. (2020) is used. Though the variable importance (VIP) and coefficients of PLS regression were defined in the past (Luedeling & Gassner, 2012), PLS results are not always unambiguous (Luedeling et al., 2013a). Therefore, Wenden et al. (2020) suggested using the same VIP and coefficient criteria as Luedeling & Gassner (2012), plus the consecutive days that coefficients were above or below the criteria to make it more consistent to start with. Still, the PLS regression does not always work, especially when it comes to the chilling period (Luedeling et al., 2013a). In that case, we also visualized the PLS-generated patterns to inspect the chilling and forcing periods with expert judgment. PLS analysis does well in a situation with many observations and a clear

normal baseline temperature pattern. Therefore, applying PLS to conifer data may still have a long way to go. Though using PLS regression is a departure from more traditional approaches and requires specific criteria to be met, it can more objectively identify the start and end dates of chilling and forcing phases (Wenden et al., 2020). Despite these limitations, the application of PLS regression in this context is well supported by recent literature (Benmoussa et al., 2017; Fernandez et al., 2021; Guo et al., 2015a; Guo et al., 2013; Guo et al., 2015b; Luedeling, 2012; Luedeling et al., 2013b, 2013a; Luedeling & Gassner, 2012; Martínez-Lüscher et al., 2017; Wenden et al., 2020). We addressed the aforementioned limitations by combining PLS with a sound ecological theory, guiding us to interpret the results, visualizing PLS-generated patterns, and including as much data as possible. Results are biologically sound, consistent with other studies, and imply potential testable hypotheses for further investigation.

### 5.2.2 Pollination study in conifers

Our understanding of pollination in conifers has advanced rapidly in the past two decades, but it still falls behind our knowledge of this process in flowering plants. To better explain how temperature responses of pollen performance vary among conifer species and within species under climate change, I suggest several future directions of conifer pollen study. (1) Studies that focus on the pollination mechanism of conifers. Three traits are correlated in conifer pollination mechanisms: 1. ovule orientation at the time of pollination; 2. pollination drop exuded from the micropyle; 3. pollen buoyant or sinking (Owens et al., 1998). Different families or genera of conifers have different traits that determine the pollination mechanisms. Other environmental factors not included in this study will also affect different traits, which must be studied further. For example, higher relative humidity and precipitation are important for successful pollination of the species where pollination drops are absent. (2) The genetic control and biochemistry involved in conifers' pollen germination process. Fernando et al. (2005) mentioned that the pollen tube is an ideal experimental system to explore these areas where we know little about conifers. (3) The influence of pollen-pollen interaction on fertilization abilities. Parantainen & Pasonen (2004) found that direct pollen-pollen interactions can influence pollen fertilization ability when pollen from different donors and origins germinate on

the same flower and can be more pronounced among specific genotypes. Conifer pollen is wind-disseminated, and it is very likely that many pollen from different donors are dispersed to the female cones. How these pollen grains interact with each other will have significant effects on gene flow and population dynamics. (4) Studies that focus on pollen's plastic response on the individual level. This would contribute to the knowledge of plant populations' evolutionary adaptiveness in a changing climate (Steinacher & Wagner, 2012) and help to improve understanding of the species' ability to persist in the future. (5) A pollen germination and tube growth study in vivo. The environments in vivo and in vitro are quite different, and the involvement of ovules may change the pollen's performance in germination and tube growth. A link or transform between pollen germination in vivo and in vitro would be very useful in pollen study since it is difficult to make observations and apply temperature treatments on large trees. (6) Another potential study not related to pollen is the temperatures in the pollen-collecting sites. We have HOBO temperature data that could be used to inform the accuracy of the downscaled ClimateNA data.

### 5.2.3 Studies on abiotic and biotic effects on conifer seed germination and phenotypic plasticity

Some limitations were shown in the seed germination study. The observed germination percentage,  $GR_{50}$ , and optimal temperatures varied among elevations, but no specific patterns or elevational trends existed. We used seeds that were not only from different elevations but also across a wide range of the Western United States, making temperature confounding with many other factors. To account for this, we used a PCA to characterize the climatic differences among these sites and correlate climate with seed germination traits. However, additional factors relating to seed quality created other errors; for example, seed age ranged from two years to more than 40 years old. Due to the small sample sizes of different seed ages and elevations of individual species, we could not analyze the seed age effect on the individual species from different elevations. Seed ages are related to the seed germination percentages, and additional variation in seed quality related to collecting, handling, and storage also cannot be quantified. All of these factors may dilute the environmental and elevational trends. Understanding this variation is important for predicting and managing plant population

dynamics and for conserving plant diversity in the face of environmental change. However, many other factors impact seed germination traits related to unknown seed quality characteristics. We recommend seed collection specifically for research to minimize other unwanted factors in the future. Foresters have long known that using locally produced seeds promotes the success of reforestation from seeds (Johnson et al., 2004). However, because of rapid climate change, people can no longer assume that locally sourced seeds are always the best or appropriate option (Havens et al., 2015). Plants are responding to changing climates through plasticity, adaptation, and migration, and knowing how this may influence seed-sourcing decisions is essential for successful restoration in the future. Temperature is one of the most critical environmental factors that regulate seed germination in the field, but not the only factor. Continued collection of seeds from diverse environments and studying abiotic (e.g., temperature, precipitation, soil moisture content, etc.) and biotic (e.g., competition, herbivory, etc.) effects on seed germination are critical to improving our understanding of how the early stage of plant life will respond to climate change. This information can help identify the most crucial factors for species regeneration and aid in seed transfer, assisted migration, and designing effective refugia as climate change progresses.

#### 5.2.4 Implementation of temperature dependences of conifer reproduction to the species distribution model

I propose an improved process-driven species distribution model incorporating temperature effects on reproductive success and recruitment to enhance projective accuracy and answer more questions in the future. Moving-habitat models (MHMs) with integrodifference equations to model population density under climate change (Harsch et al., 2014; Kot & Phillips, 2015; Zhou & Kot, 2011, 2013) allow us to bring all factors and climate-change velocity together to predict the rate of change under different scenarios. For example, the temperature dependence of pollen germination and tube length can be used to adjust the current natural reproductive rate to the rate under specific temperature conditions in the future. Similarly, the temperature dependence of seed germination can be used to adjust the

current recruitment rate to the rate under specific temperature conditions. Climate-change velocity can be obtained from other research.

MHM will be used to model population density under climate change (Harsch et al., 2017). Integrodifference equations (IDEs) will be used in MHM, which model space as continuous and time as discrete. IDEs have been used in many disciplines, including climate-change biology (Harsch et al., 2014; Kot & Phillips, 2015; Zhou & Kot, 2011, 2013). Here is a basic MHM using the IDE,

$$n_{t+1}(x) = \int_{-\frac{L}{2}+ct}^{\frac{L}{2}+ct} k(x-y) f[n_t(y)] dy$$

The function  $n_t(x)$  describes the population density at time  $t$  at every location  $x$  along the one-dimensional habitat ( $L$ ). The habitat moves by a fixed increment of  $c$ , the shift speed in suitable climatic conditions. At each point  $x$  along the habitat ( $L$ ), the local population  $n_t(x)$  produces offspring according to some growth function  $f[n_t(x)]$ . Seeds disperse according to the dispersal kernel  $k(x-y)$ . For a fixed seed source location  $y$ ,  $k(x-y)$  is the probability density of a seed dispersing to destination  $x$ . Parameterizing this model involves estimating the dispersal kernel, growth function, habitat size, and velocity of climate change. The first three components are not unique to MHMs and belong to a much larger literature of IDEs and other population-dynamic models (Harsch et al., 2017). Several dispersal kernels can be tried, depending on the suggested dispersal and growth function and dispersal statistics from the literature. Applying the entire species distribution range for habitat size  $L$  is a good start in future studies. The pollen viability and seed germination under different temperatures can adjust the growth function's reproduction, growth, or survival rates. A two-stage structure (juvenile and adult) model is also a reasonable choice because trees are long-lived and do not reproduce for the first several years.

### 5.2.5 Studies on temperature effects on seedling mortality and growth

In 5.2.4, results from seed germination tests were proposed to estimate the recruitment rate in the species distribution model. However, the recruitment is affected by many factors.

Therefore, conducting temperature experiments on seedling mortality and growth to strengthen the recruitment estimation is necessary. Understanding the mechanisms of differing tree growth and survival to extreme temperature events is critically important for understanding how tree species will be affected by climate change. Therefore, incorporating the seedling mortality and growth response to high temperature into the recruitment estimation is important because extreme heat events can impact various seedling functions at the cellular, leaf, and whole plant levels (Teskey et al., 2015) and eventually affect the recruitment rate. In addition, high temperatures significantly increase the severity of drought stress. Thus, exploring drought at the same time is also required because it is a more severe stressor than heat (Bauweraerts et al., 2014). The temperature treatments can include ambient temperature (control), ambient +3°C, a heat wave elevating ambient temperature by 6°C every other week, and a heat wave elevating ambient temperature by 12°C every four weeks. Each applied heat wave lasted one week, and treatment schemes can be repeated throughout the growing season. Many morphological and physiological traits can be collected during the experiment. The results can be applied to the species distribution model to regulate recruitment and growth rates.

#### 5.2.6 Studies on genetic variability

In Chapter 4, germination rates showed significant variation, but the environments of seed source locations did not completely account for differences in intraspecific germination rates, implying a genetic component may drive seed germination rates in addition to the environment. This genetic component can also be applied to the study of pollen viability. Genetic variation in response to heat has received limited study in trees, but such variation exists within species and could be exploited to improve heat stress tolerance (Teskey et al., 2015). A study has shown that incorporating intrapopulation genetic diversity and phenotypic plasticity in species distribution models significantly altered their outcomes (Benito Garzón et al., 2011). Therefore, I propose to include a genetic analysis that can identify intraspecific DNA sequence variations linked to adaptation in future studies to test if the genetic variability will affect population persistence.

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