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Phenotypic Variation in Climate-Associated Traits of Red Spruce (*Picea rubens* Sarg.) along Elevation Gradients in the Southern Appalachian Mountains

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ABSTRACT

Red spruce (*Picea rubens*) is a long-lived tree species that thrives in cool, moist environs. Its ability to adapt to rapidly changing climate is uncertain. In the southern Appalachian Mountains, red spruce reaches its greatest abundance at high elevations, but can also occur across a range of mid and lower elevations, suggesting the possibility of a correlation between genetic variation and habitat. To assess clinal phenotypic variation in functional traits related to climate adaptation, we collected seed from 82 maternal sib families located along replicated elevational gradients in the Great Smoky Mountains National Park, TN (GSMNP) and Mount Mitchell State Park, NC (MMSP). The percentage of filled seeds and seed mass increased with elevation, indicating that successful pollination and seed development was greatest at the highest elevations. Seedlings sourced from GSMNP displayed a strong relationship between elevation and bud set when grown under common garden conditions. High elevation families set bud as many as 10 days earlier than low elevation families, indicating adaptation to local climate. Across parks, no effect of elevation was noted for bud flush. Our results demonstrate that red spruce in the southern Appalachian Mountains displays clinal variation in bud set that may reflect local adaptation to climate, although this varied between the two parks sampled. We suggest that genetic adaption of red spruce to different climate regimes, at both local and broad spatial scales, is in need of more intensive study, and should be carefully considered when selecting seed sources for restoration.

Key words: adaptation, phenology, *Picea rubens*, red spruce, southern Appalachians

INTRODUCTION

Climate change is likely to pose significant threats to many endemic or regionally restricted species in Appalachian forest ecosystems. Among the most vulnerable species are those inhabiting the high elevation spruce-fir forests of the southern Appalachians (Virginia, North Carolina, and Tennessee), as these communities form mountain-top "sky islands" where upslope migration in response to climate change is limited. For this reason, migration on its own is unlikely to be a sufficient response for isolated high-elevation populations facing climate change, and a response to selection on existing genetic variation in climate-adaptive traits will likely be necessary (Aitken et al. 2008, Vitt et al. 2010). Such genetic variation, if present, also has the potential to play an important role in restoration efforts, and has historically been used in other regions to delineate "seed zones" used by

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foresters and restoration ecologists in guiding climate-informed planting strategies (McKenney et al. 2009, Bower et al. 2014, Thomas et al. 2014). Thus, there is a critical need to obtain estimates of genetic variation in functional traits related to climate adaptation in foundational species within the high-elevation spruce-fir ecosystem.

Red spruce (*Picea rubens* Sarg.) is an iconic conifer species of high elevation forests in the southern Appalachians, where it provides critical habitat to a variety of other plant and animal species (Rentch et al. 2007, Fortney et al. 2015, Diggins and Ford 2017, Walter et al. 2017). Red spruce thrives in cool, moist environments; there are concerns that it may be unable to adapt to land-use alteration and climate change (Iverson et al. 2008, Beane 2010, Andrews 2016). Pollen records from southern Appalachian bog sediment cores show that *Picea* spp. became regionally restricted to the higher elevations of the Cumberland and Allegheny Plateaus from 9,000 to 4,000 years ago as spruce migrated to higher latitudes and elevations in response to the warming climate of the mid-Holocene (Delcourt and Delcourt 1984). The current populations of red spruce in the southern Appalachians are locally isolated on high elevation ridgetops, disjunct from the more abundant and well-connected northern populations in New York, New England, and the Canadian Maritime provinces (Figure 1A). Heavy logging followed by severe fires in the early 20th century resulted in >90% reduction in spruce-fir forests in the southern Appalachians (Korstian 1937). As such, red spruce is the focus of multi-agency restoration efforts aimed at restoring red spruce forests to high elevation landscapes in the central and southern Appalachians, i.e., the Central Appalachian Spruce Restoration Initiative (CASRI; **www.restoreredspruce.org**) and Southern Appalachian Spruce Restoration Initiative (SASRI; **www.southernspruce.org**).

Considering that the southern Appalachians were not glaciated and *Picea* species were long-term occupants of glacial refugia in this region (Davis 1980), southern red spruce populations may have evolved genetic variation in climate-adaptive traits associated with growth and phenology. Southern populations also occur across a range of elevations (900 m to 2000 m) resulting in steep climatic gradients in growing season length, sometimes over relatively short geographical distances (Brown 1941, Crandall 1958, Schofield 1960, Cogbill and White 1991). As a result, red spruce located along elevational gradients likely have experienced historic and ongoing divergent selection pressures in response to varying local climate conditions, but it is unknown whether this has led to genetic and phenotypic differentiation in climate-adaptive traits. Identifying the potential genetic capacity of red spruce to respond to climate change is critical to informing conservation strategies not only in the southern Appalachians but also across its entire geographical range. Climate change has put southern populations at risk of local extinction as upslope migration opportunities are limited. Considering that red spruce achieves its greatest growth potential and stem quality in the southern Appalachians (Korstian 1937, Nowacki et al. 2010), these populations could provide valuable seed sources in light of continued climate change in the northern part of the range.

In this study, we test for phenotypic variation in seed and seedling traits related to climate adaptation in southern Appalachian red spruce. We collected open-pollinated seed from maternal half-sib families from multiple sites distributed along replicated elevation gradients in North Carolina and Tennessee. Using elevation as a proxy for climate, we tested for a relationship between source elevation and early life history traits related to seed quality, vegetative bud phenology and growth that form important components of seedling performance under varying climate conditions. Our results provide a first glimpse at the presence of climate-adaptive phenotypic variation along elevation gradients in southern Appalachian red spruce, and call for further study of genetic variation and its potential for guiding seed selection for restoration of red spruce in this imperiled ecosystem.

METHODS

Seed Collection and Analysis

Cones were collected from 82 red spruce trees growing naturally at elevations ranging from 1036 to 1988 m in North Carolina and Tennessee from 12–16 September, 2016 (Figures 1B, 1C, respectively;

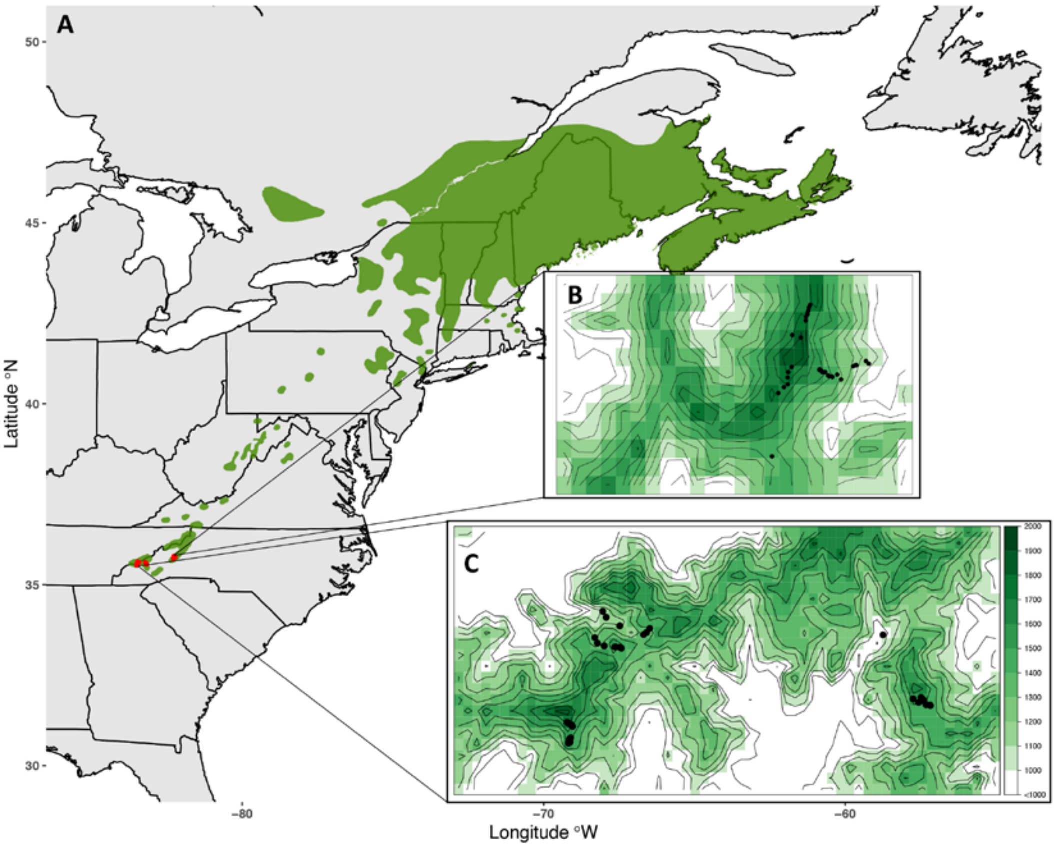

Figure 1. Natural range of red spruce[1] in eastern North America (A). Insets show location of mother trees and topographic features at Mount Mitchell State Park (B), and Great Smoky Mountains National Park (C).

Table 1). As cones were open-pollinated, all seeds from a given mother tree are considered maternal families (hereafter, "families") consisting of a potential mix of full and half-sibs with the same mother but unknown fathers. Collections were made from trees thought to be naturally regenerated using documentary evidence and cues such as uneven age stand structure and irregular spatial distribution i.e. not row planted. Cone collections were centered on Mount Mitchell State Park (MMSP) and Great Smoky Mountains National Park (GSMNP), as they are each populated with red spruce across a broad elevation and climate gradient (Figures 1B, 1C; Table 1). The three sites at MMSP were within 5 aerial km of each other. The high elevation Deep Gap Trail spans the ridgeline from Mount Mitchell to Mount Craig and beyond to Big Tom. Disturbance history maps of Mount Mitchell developed by Pyle and Schafale (1988) indicate that the west side of the ridge was uncut as of 1916, while the east side was cut and burned. There was no evidence of planting in this area and collections were made primarily from second growth trees. The Commissary Ridge Trail that leads to Camp Alice was proximal (~1 km), but not immediately adjacent to areas that were previously cut and planted with a variety of species in the 1920s (Minckler 1940). Cone collections were primarily from naturally regenerated trees following harvest. The Mitchell trail from 1711 m to ~1400 m extends beyond the disturbance history maps by Pyle and Schafale (1988), but the trees are markedly older and likely survived by being too small at the time of the destructive

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[1] Public domain image. USGS Geosciences and Environmental Change Science Center: Digital Representations of Tree Species Range Maps from "Atlas of United States Trees" by Elbert L. Little, Jr. 1971

Table 1. Collection of seed cones from red spruce naturally occurring along elevational gradients in the southern Appalachians.

State	Site	Families Collected (#)	Families Propagated (#)	Elevation (m)	MAT[1] (°C)	Latitude	Longitude
NC	Commissary Ridge Trail, MMSP[2]	6	3	1736–1840	7.8–7.1	35.752	-82.276
NC	Deep Gap Trail, MMSP	10	5	1897–1988	6.7–6.0	35.781	-82.260
NC	Mitchell Trail, MMSP	19	8	1198–1711	11.5–7.9	35.754	-82.245
NC	Andrew's Bald Trail, GSMNP[3]	19	6	1734–1842	7.8–7.0	35.547	-83.494
NC	Heintooga Ridge Road, GSMNP	10	7	1036–1627	12.6–8.5	35.575	-83.173
TN	New Found Gap, GSMNP	4	2	1314–1330	10.7–10.6	35.624	-83.430
TN	Road Prong Trail, GSMNP	14	7	1100–1617	12.2–8.0	35.615	-83.457

[1] Mean annual air temperature (MAT) was estimated using data (1998-2018) from four land-based stations: Mount LeConte, TN (1979 m), Mount Mitchell, NC (1902 m), New Found Gap, TN (1536 m) and Cherokee, NC (1036 m). After gap-filling, linear regression was used to predict temperature by elevation: MAT=19.75–0.0069 * elevation (m); R^2=0.99, p=0.001. Data were accessed from NOAA Climate Data Online, August 6, 2018 (**https://www.ncdc.noaa.gov/cdo-web/**).

[2] Mount Mitchell State Park (MMSP)

[3] Great Smoky Mountains National Park (GSMNP)

harvesting. At GSMNP, three sites were within 8 aerial km, while the remaining site (Heintooga Ridge Road) was, at its most distant point, 29 km away. Pyle and Schafale (1988) produced a detailed map of the disturbance history near Clingman's Dome and the vicinity of the Road Prong trail. Our collections from the Andrews Bald trail were from naturally regenerated second growth trees adjacent to an older uncut stand, to the north. Road Prong Trail had not been harvested and featured very large, old trees. Collections from both the New Found Gap Road and Heintooga Ridge Road were made from near the roadside. GSMNP had no documentary evidence that these areas had red spruce plantings.

After collection, cones were dried and the seeds were extracted and processed at the USDA Forest Service National Seed Laboratory in Dry Branch, Georgia. A subset of seeds from each family was counted and weighed to yield mean seed mass prior to separating filled from unfilled seed, and 200 seeds were imaged with a Faxitron Ultra Focus x-ray system (Faxitron Bioptics, LLC, Tucson, Arizona) to estimate the percentage of filled seed out of the total seed count for each family (Figure 2). Seeds are considered filled if the x-ray indicates they contain all tissues and morphological features required for germination. Percentage of filled seed is an indirect indicator of viability, but is not as definitive as a measure of germination, embryo growth, or metabolic activity would be. The seed lot for each family was then cleaned with a blower to remove debris and lightweight material such as unfilled seeds, followed by another subset of seeds per family counted and weighed to yield clean seed mass. The seeds were then kept refrigerated at 4°C until planting.

Germination and Propagation

A subset of 38 red spruce families were selected for germination and propagation to maximize the spatial distribution and elevation gradient at each park (Table 1). The difference in latitude between collection sites within each transect was minor (<0.25°). Twenty filled seeds from each family were placed in deionized water to imbibe for germination. After 24 hours, the seeds were drained and placed on moist blotter paper in Petri dishes and lightly sprinkled with sand to aid wicking. The dishes were incubated in an Achieva precision tabletop light/dark germinator

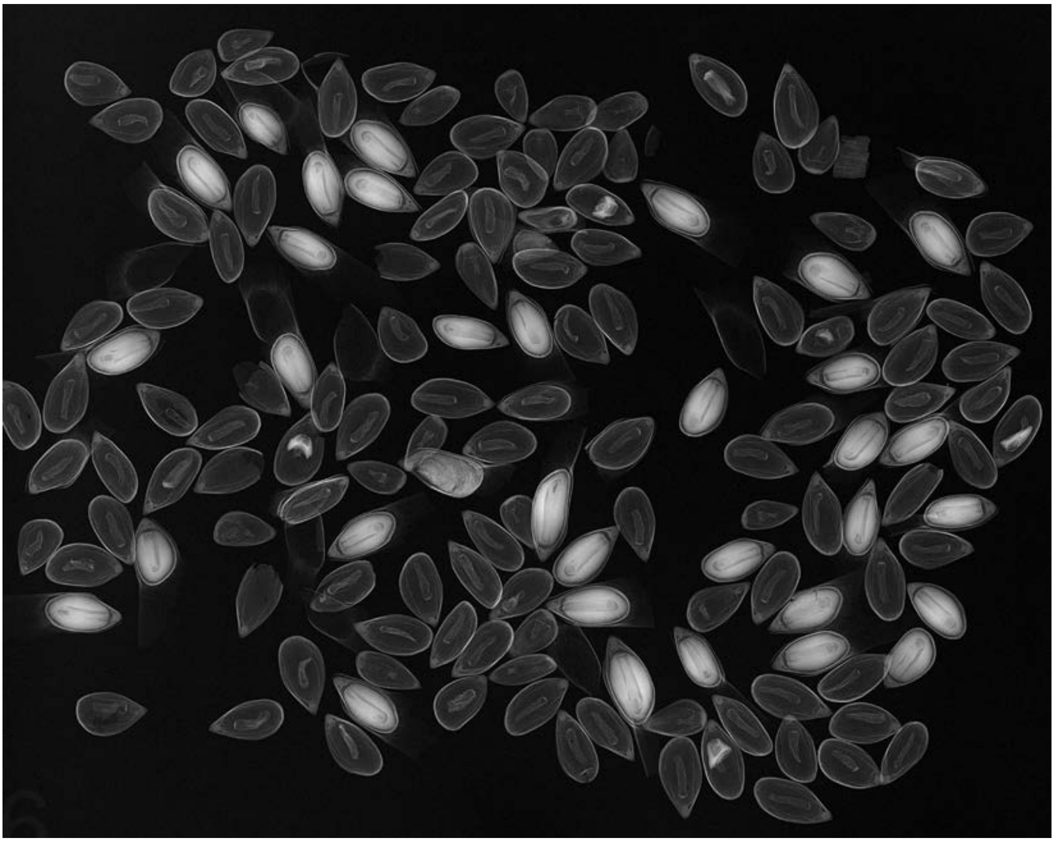

Figure 2. X-ray of red spruce seeds. Filled seeds appear white and details of embryonic structure are evident, while unfilled seeds remain dark and empty.

(Seedburo Equipment Co., Des Plaines, Illinois) in darkness for 16 hours at 20°C followed by 30°C in light for 8 hours (AOSA 2016). Germination was observed every 2–3 days, and recorded when the emerging radicle was 2–3 mm in length. Seeds were germinated in two rounds beginning on 17 January, 2017, and 10 February, 2017, as they became available from the National Seed Laboratory. Germinated seeds were transplanted from the dish to Ray Leach conetainer pots (model sc10) in a peat-perlite-vermiculite soilless media. The seedlings were maintained in a Conviron PGR15 growth chamber with supplemental light (8 hours at 18°C dark followed by 16 hours at 25°C light) before being moved in May 2017 to an outdoor nursery under shade cloth (50% light transmission) at the University of Vermont (UVM) greenhouse. While outside, the seedlings were exposed to ambient temperatures for Burlington, Vermont, were watered daily and received periodic water soluble fertilizer applications at a concentration of 150 ppm (19-3-18; N-P-K ratio). After seedlings had naturally set bud outdoors in fall 2017, they were overwintered in a cold room of the UVM greenhouse minimally heated to maintain above freezing temperatures with no supplemental lighting. In April 2018 they were again placed outdoors under shade cloth and allowed to accumulate growing degree days leading up to spring bud flush.

Phenological and growth measurements

To quantify genetic variation in vegetative phenology and growth among families when exposed to common environment conditions, we measured the onset of dormancy as bud set and the release of spring dormancy and the start of active shoot growth as bud flush. Bud set was determined by observing individual seedlings every 2–3 days during fall 2017 and recording the Julian date when bud scales were clearly visible on the terminal shoot. After the terminal bud had set, the length of the stem covered by live foliage was recorded as live crown. Bud flush was determined in spring 2018 by observing individual seedlings every 2–3 days, and recording the Julian date that bud scales had broken and newly emerged needle tissue was evident.

Statistical Analyses

We used linear mixed-effects and generalized linear mixed-effects modeling fit by maximum likelihood to test for significant variation in bud phenology, growth, filled seed, and germination as a result of collection elevation, park, and the interaction of elevation and park. In each model, family nested within park was included as a random effect to account for multiple seedlings per family. Likelihood ratio tests were used to determine significance of the random effect when family was removed from the model. In the analysis of seedling growth, we included the round in which seedlings were germinated as an additional fixed effect. Seed mass was collected at the family level, hence we used linear models with collection elevation, park, and the interaction of elevation and park as fixed effects. Pearson's correlation coefficient was used to define the bivariate correlation between seed mass and germination percentage by park. Analyses were performed using R version 3.4.0 (R Core Team 2017) with packages lme4 (Bates et al. 2015) and lmerTest (Kuznetsova et al. 2017). Outlier detection was accomplished using the Cook's Distance statistic, which combines information about the residuals with the degree of leverage that an observation has on the regression equation (Cook 1977).

RESULTS

Seed properties

The mass of clean, filled seeds was highly variable (range 1.7 mg–5.3 mg per seed) and increased significantly with elevation (Table 2, Figure 3A). The percentage of filled seed determined by radiographic imaging showed a marginally significant positive trend with elevation (p=0.0703; Table 2). When data from both parks were combined and analyzed with linear regression, 8% of the variation in filled seed was explained by elevation (p=0.0092; Figure 3B). For both GSMNP and MMSP, the very low percentages of filled seed at elevations <1400 m are driving the positive linear relationship (Figure 3B). Germination percentages of cleaned, filled seed varied widely after 4 weeks (5%–95%; mean across families = 45%). Significant effects of park (p<0.0001) and park*elevation (p=0.00028) revealed a strong negative linear relationship between germination and elevation of seed origin at MMSP, but not at GSMNP (Table 2, Figure 3C). There was no significant correlation between seed mass and germination at either GSMNP (p=0.2840) or MMSP (p=0.8813). Significant variation among families was found for filled seed (p<0.0001) and germination percentages (p<0.0001).

Phenology

Significant effects of elevation were identified for bud set (p=0.0108), but not bud flush (p=0.6724) (Figure 4A, 4B; Table 2). When grown under common environmental conditions, high elevation seedlings from GSMNP set bud as much as 10 days earlier than seedlings from low elevation sources. Elevation explained 30% of the variation in fall bud set at GSMNP (Figure 4A). Two potential outliers at low elevation were observed (Cook's Distances of 1.25 and 1.6), indicating they were highly influential on the regression equation, whereas the remaining observations had minimal leverage as outliers (Cook's Distances of <0.2). When those two observations are removed, the relationship between elevation and bud set strengthened considerably at GSMNP (Figure 4A), where elevation accounted for 64% of the variation in bud set. It is notable that a strong relationship between elevation and bud set was not found at MMSP (Figure 4A). There was not a significant effect of family for bud set (p=0.2471) or bud flush (p=0.6404), indicating a lack of significant genetic variation among families beyond what is already explained by the elevation gradient (e.g., for bud set).

Table 2. Summary of general and mixed-effect linear models analyses describing the effect of elevation, park, and the interaction of park*elevation on seed traits, seedling growth, and bud phenology. The fixed effect "round" represents two different planting dates and was only included for the dependent variable live crown. Family was treated as a random effect and significance was tested with likelihood ratio tests. Some variables required re-scaling elevation to mean = 0, SD = 1 to achieve model convergence. *p* values significant at the 0.05 level are indicated with bold font

Model Effects	Estimate[1]	Std. Error	t or χ^2	*p*
Seed Mass				
Elevation	1.63 E-6	3.70 E-7	4.436	**<0.0001**
Park	1.24 E-3	1.05 E-3	1.187	0.2392
Park*Elevation	-6.10 E-7	6.20 E-7	-0.975	0.3330
Full Seed				
Elevation[2]	0.2947	0.1628	1.810	0.0703
Park	-0.3341	0.2508	-1.332	0.1828
Park*Elevation	0.2277	0.2546	0.894	0.3713
Family	1.1560	–	2866.7	**<0.0001**
Germination				
Elevation[2]	0.2794	0.1613	1.732	0.08319
Park	1.2392	0.2822	4.391	**<0.0001**
Park*Elevation	-1.0795	0.2974	-3.629	**0.00028**
Family	0.3370	–	18.885	**<0.0001**
Bud Set				
Elevation	-0.0089	0.0034	-2.638	**0.0108**
Park	-15.5522	7.5912	-2.049	**0.0489**
Park*Elevation	0.0099	0.0047	2.119	**0.0414**
Family	1.9250	–	1.340	0.2471
Bud Flush				
Elevation	0.0010	0.0024	0.425	0.6724
Park	3.6345	5.0453	0.720	0.4786
Park*Elevation	-0.0020	0.0031	-0.626	0.5369
Family	0.4812	–	0.218	0.6404
Live Crown				
Elevation[2]	-0.1145	0.1127	-1.015	0.3160
Round	1.6735	0.1864	8.980	**<0.0001**
Park	0.0260	0.1726	0.150	0.8820
Park*Elevation	0.2172	0.1604	1.353	0.1860
Family	0.1060	–	6.960	**0.0084**

[1] For Elevation, Park, and Round, estimates are the fixed effects model parameters; for Family, estimates are the standard deviation of the random effect.

[2] Test statistics report t-tests for fixed effects, and the χ^2 likelihood ratio for random effects

Growth

The height of live crown was not significantly related to elevation (p=0.316) or park (p=0.882), though planting round (p<0.0001) was highly significant (Figure 4C; Table 2). Variance among families was highly significant for live crown (p=0.0084), suggesting a large amount of genetic variation for early seedling growth. At GSMNP there was a significant linear relationship between bud set and live crown length (p=0.0132); however this relationship was not observed for MMSP (Figure 5). Considering that the extended length of the growing season prior to bud set is only 10 days, the difference in live crown may indicate slower growth rates of seedlings sourced from higher elevations.

DISCUSSION

We found significant variation in seed traits and bud phenology among red spruce families collected along an elevational gradient in the southern Appalachians. These early life history traits are known

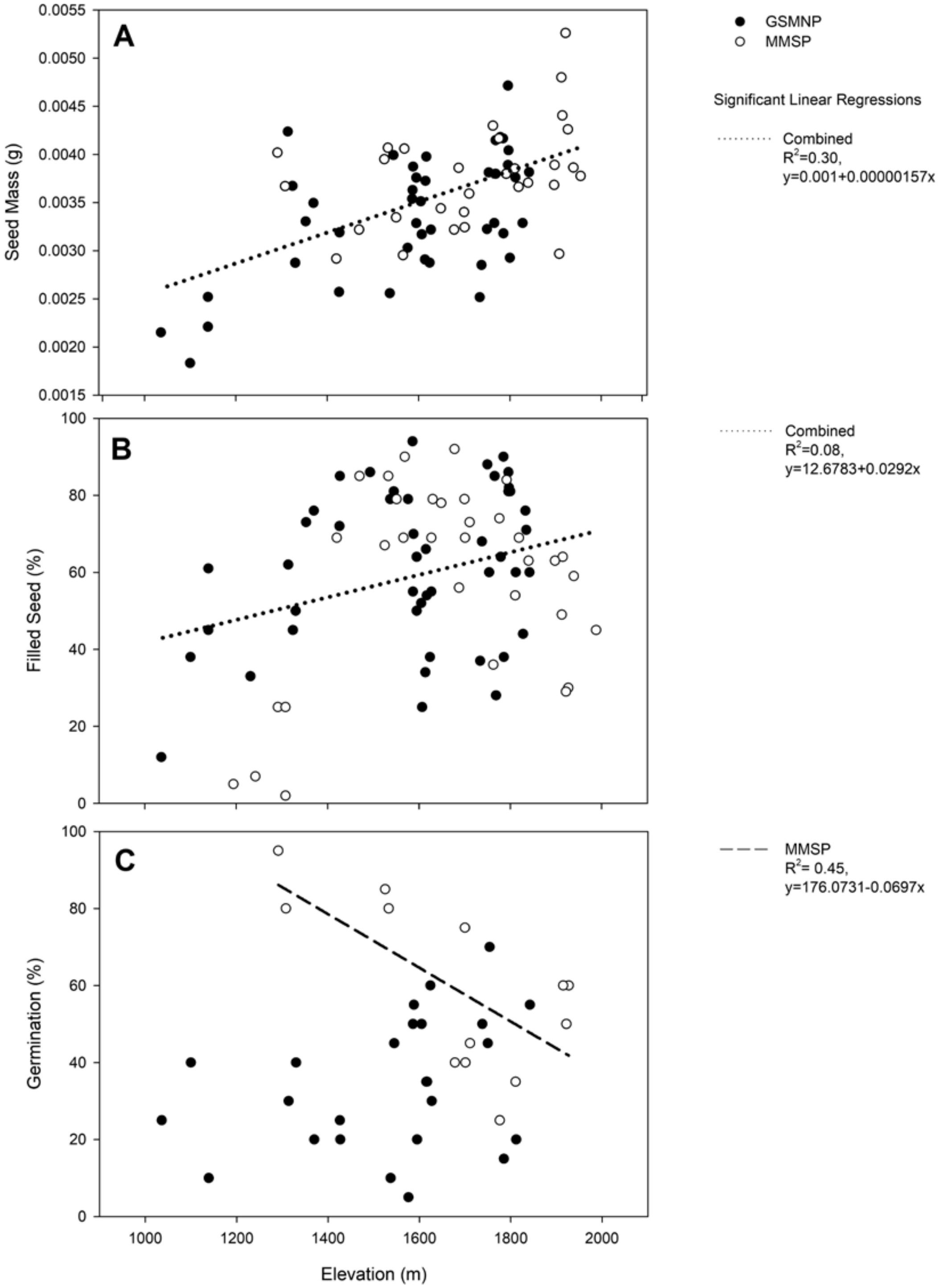

Figure 3. Mean family seed mass (n=73) (A), percentage of filled seed (n=80) (B), and percentage of seed germination (n=38) (C) of red spruce collected in Great Smoky Mountains National Park (GSMNP) and Mount Mitchell State Park (MMSP) by source elevation.

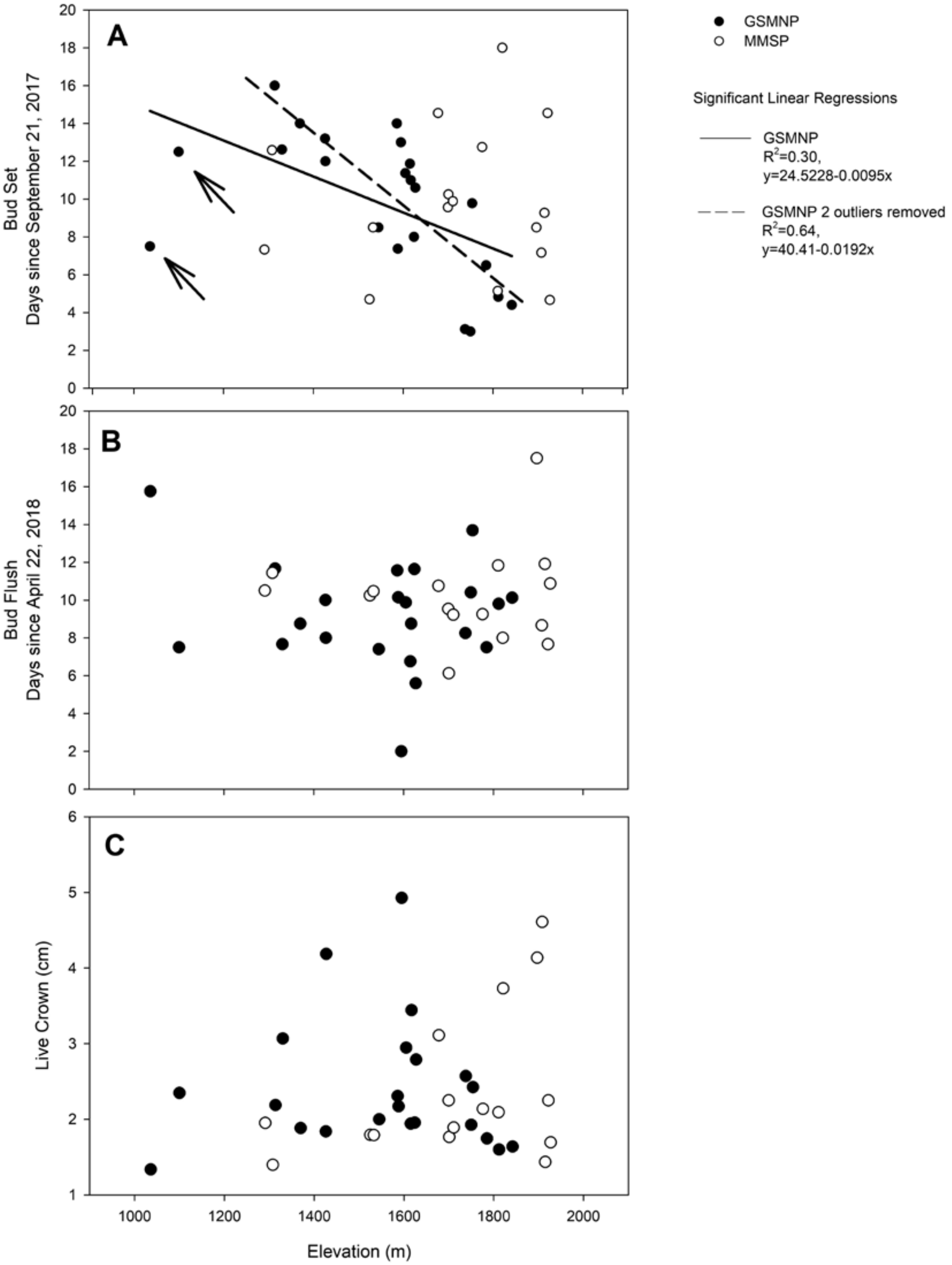

Figure 4. Mean bud set (A), bud flush (B) and live crown (C) by family (n=38) for seedlings grown in Burlington, Vermont from seeds collected in Great Smoky Mountains National Park (GSMNP) and Mount Mitchell State Park (MMSP) by source elevation. Arrows denote two outliers identified using Cook's Distance statistic (A), the regression parameters were re-calculated without the outliers for GSNMP.
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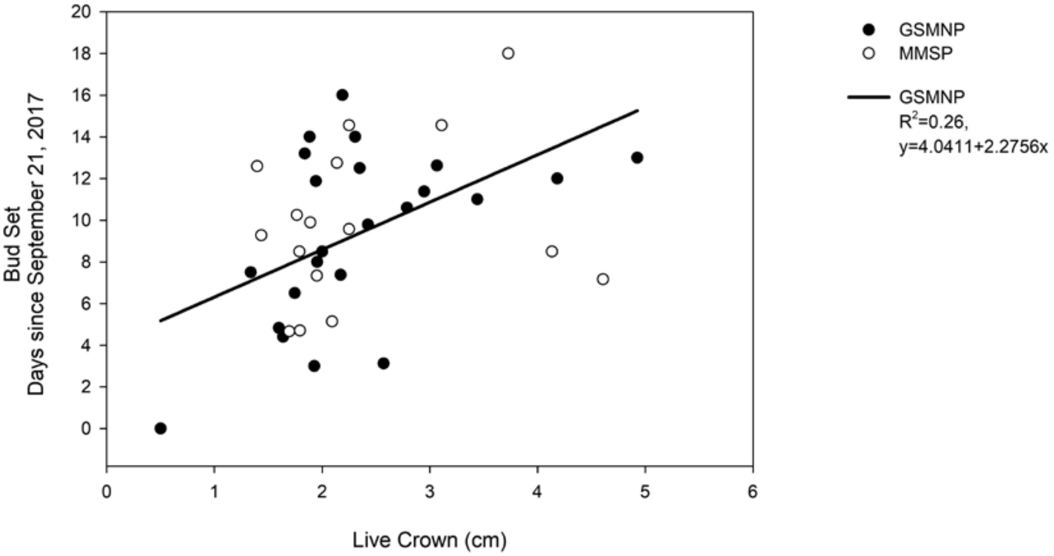

Figure 5. Relation between bud set and total live crown at dormancy in fall 2017.

to be important components of plant fitness (Westoby et al. 1996, Savolainen et al. 2007), and as such may be under divergent selection in different environments. While our breadth of sampling is moderately limited in regional scope, this study provides an important first report of potentially adaptive phenotypic variation among neighboring southern Appalachian red spruce populations.

Both seed mass and percentage of filled seed exhibited positive clines with elevation. In many montane plant species, seed size and number of seeds per plant are affected by elevation, and species display both positive and negative relationships (Oleksyn et al. 1998, Liu et al. 2013, Olejniczak et al. 2018). Oleksyn et al. (1998) found that Norway spruce (*Picea abies* (L.) H. Karst.) seed mass declined and the percentage of unfilled seeds increased with elevation in the mountains of Poland as the climate becomes colder. Similarly, black spruce seed mass and percentage of filled seed was found to be negatively correlated with elevation across Canada (Liu et al. 2013). Further, across *Picea* species, seed mass has been shown to decline with latitude (Miyazawa and Lechowicz 2004) and within species black spruce and white spruce seed mass also decline with latitude (Liu et al. 2013). Thus, one might predict that colder, higher latitude or elevation sites with shorter growing seasons would reduce seed quality. However, we observed the opposite trend with red spruce in the southern Appalachians, with the percentage of filled seed and mean seed mass increasing with elevation (Figure 3A, 3B).

The southern Appalachians lay at the trailing edge of the range of red spruce; it would be simple to directly correlate seed mass to elevation with the rationale that low elevation sites have sub-optimal climatic conditions while high elevation are considered optimal. High elevation sites may experience reduced heat stress and reduced stress related to high vapor pressure deficit, but other stresses such as air pollution (formerly), wind, rapid temperature swings, shorter growing season as well as competition from Fraser fir (*Abies fraseri*, (Pursh) Poir.) may limit growth rate, longevity and health. Mathias and Thomas (2018) found that red spruce at three sites in West Virginia (lat. 38°N) were now growing at a faster rate than at the prior peak in the early 1960's before air pollution led to sharp growth declines. This renewed growth under the present warmer atmospheric conditions as well as recent downslope migration of montane ecotones (Foster and D'Amato 2015) indicates that red spruce may tolerate warmer conditions and occupy greater "potential range" than previously thought. In addition, compared to related *Picea* species, the relationship between elevation and seed traits in red spruce suggest mechanisms other than climate, possibly reflecting Allee effects (Allee and Bowen 1932, Stephens et al. 1999) of a larger, more diverse mating population growing in more optimal environments at higher elevations. For example, some of the variation in seed traits may reflect population differences in pollination efficiency or resource availability with elevation. Empty or non-viable seeds may result from self-fertilization, inbreeding, or lack of pollen when the female cone is receptive to fertilization. In black spruce, the percentage of filled seed is directly related to the quantity of pollen grains at a given location (Caron and Powell 1989) and this would also be the expectation for other *Picea* species. While we can only speculate as to the cause of the elevation gradient on seed quality in the current study, possible explanations include: **1)** increased population density at higher elevations increasing the size and diversity of the pollen pool and offsetting pollen limitation and the probability of inbreeding depression (Mosseler et al. 2000, Rajora et al. 2000); **2)** better phenological synchrony of available mating partners at higher elevations (LaMontagne and Boutin 2007), **3)** better pollination efficiency due to favorable wind characteristics on mountain tops and ridgelines (Fall 1992), or **4)** increased drought or heat stress during the seed provisioning stage at lower elevations. While it is not possible to identify specific causes for the elevation effect on seed quality from our results, it does seem clear that seed quality is impacted at lower elevations.

Germination percentages of filled red spruce seed were much lower than reported in the literature for other regions, e.g. New Hampshire, 62–77% (Baldwin 1934), New Brunswick and Nova Scotia, >80%, more distant populations in Ontario, >70% (Major et al. 2003), and New Brunswick >95% (Butnor et al. 2018). Surprisingly, there was no relationship between germination and seed mass, but we found significant variance among families. The low mean germination percentage from the southern Appalachians (45% in the present study) could represent higher levels of inbreeding depression in the more fragmented, southern part of the range compared to the more interconnected northern part of the range. In a study comparing old-growth remnant red spruce stands in Ontario, Canada, Mosseler et al. (2003) found tall stands (with height as an indicator of fitness) acted as potential reservoirs of genetic diversity and reproductive fitness, where mean stand height was positively related to molecular measures of genetic diversity (mean number of alleles per locus and percent of polymorphic loci), and inversely related to the frequency of rare and possibly deleterious alleles. Given a lack of recent land use history in the old-growth stands studied by Mosseler et al. (2003), the height-diversity relationship suggests that genetically diverse populations have favorable growth attributes compared to less diverse stands, perhaps because they are better able to avoid the negative effects of inbreeding depression (Mosseler et al. 2003). In our study, germination was highest at Mitchell Trail (71%) and Deep Gap Trail (58%), but all other populations were below 50%. The Road Prong population in GSMNP was primarily original forest with some of the largest mother trees in our study, yet mean germination was only 28%. Additional research into the genetic diversity and rate of inbreeding in red spruce using molecular markers and common garden studies is underway, and will improve our understanding of the causes of fitness trait variation in these remnant populations.

Common garden growing conditions in our study revealed a strong elevational cline in bud set, with earlier bud set of GSMNP red spruce seedlings from high elevation families compared to low elevation families. Because these differences exist when all seedlings were experiencing the same day length and temperature regimes, these differences likely reflect underlying genetic variation for bud set along the elevational gradient. However, maternal environmental effects or epigenetic influences on seedling traits may also be present, and could contribute to differences in early seedling growth or phenology among families collected at different elevations (Herman and Sultan 2011). Earlier bud set for trees adapted to shorter growing seasons has been observed in many other tree species, and is consistent with bud set evolving in response to local selection pressures (Mergen 1963, Johnsen et al. 1988, Oleksyn et al. 1992, Johnsen et al. 1996, Chmura 2006). Early bud set at higher elevations may be advantageous in order to terminate growth and acquire cold hardiness before freezing conditions arrive.

It is interesting that red spruce trees in close geographic proximity at MMSP, that experienced the same photoperiod and similar elevation gradient (800 m) as GSMNP, did not demonstrate strong bud set variation with elevation. One possibility is that this represents the effects of prior land use disrupting local adaptation. In the early 1900's the majority of merchantable red spruce at MMSP were cut, followed by experimental reforestation with planted red spruce among many other species (Korstian 1937, Minckler 1940, Minckler 1945, Wahlenberg 1951, Speers 1975). Dr. Clarence Korstian, who played a central role in designing the planting trial was a strong advocate for planting local seed sources (Korstian 1937) and later documentation of the experiment indicate that the red spruce were propagated from local sources (Speers 1975). Despite being "local", it is uncertain whether precise pairing between seed source and planting elevation occurred. The relatively recent introduction of red spruce genotypes from a variety of elevations could explain the lack or dilution of clinal variation along the elevational gradient at Mount Mitchell.

There were no significant relationships between elevation and bud flush from either the GSMNP or MMSP sources, despite notable differences in mean annual air temperature (MAT). The elevational cline in bud set but not flush likely reflects greater genetic determination of bud set whereas bud flush may be more environmentally plastic. Given our results, a greater selective pressure seems to exist for bud set versus bud flush along fine-scale environmental gradients. Both traits evolve as bet-hedging strategies between maximizing the period available for carbon acquisition with the need to avoid tissue damage caused by early or late freeze events. In many temperate and boreal trees, shoot elongation stops and dormant buds form in response to decreasing day length, prior to the arrival of potentially damaging cold temperatures; hence bud set is largely controlled by photoperiod, while bud flush is stimulated by the accumulation of warm temperatures in spring (Wareing 1956, Olsen et al. 2014). At the fine spatial scale of our sampling, red spruce families collected from different elevations experience highly similar photoperiods, so differences in bud set must reflect response to a different underlying cue, possibly temperature.

Similar results as ours were also found in a study of Norway spruce phenology in the Beskidy Mountains of Poland; 23 high elevation seed sources within a 12 by 18 km area experienced early onset of dormancy (800 m max) compared to low elevation (540 m min), though there were no differences in bud flush (Chmura 2006). In contrast, Johnsen et al. (1996) found that black spruce from colder, high latitude (63°) sources initiated growth earlier than those from low latitude (45°) sources when grown in common gardens, as well as exhibited differences in photosynthetic rates. Rossi and Isabel (2017) used MAT to characterize populations within more narrow latitude ranges, finding black spruce sources from colder areas initiate growth earlier than locations with higher MAT when grown together in common gardens.

Red spruce has been generally described as having low genetic diversity that could limit its ability to respond to changes in climate (Dehayes and Hawley 1992). Past assessments based on broad geographic sampling have revealed little clinal variation in morphometric traits (Gordon 1976) or in growth and survival (Fowler et al. 1988). In GSMNP, we found strong clinal variation in bud set consistent with an adaptive response to climate differences across an elevation gradient. Phenological adaptation of red spruce from low elevations in the southern Appalachians could become beneficial in future climate scenarios. Specifically, later bud set to delay growth cessation would be advantageous in a warmer climate. Eriksson et al. (1978) found strong genetic control of bud phenology in Norway spruce and through inter-provenance breeding programs, progeny could be prescribed for specific environmental conditions. Considering that past logging has left a significant amount of otherwise suitable red spruce land area vacant in the southeastern United States (Walter et al. 2017), there are still opportunities for breeding and restoration despite negative climate predictions for the species' range (Iverson et al. 2008, Koo et al. 2015). A thorough understanding of genetic variation for climate-adaptive functional traits should constitute a key component of this effort. Our study provides a first step towards this goal for southern Appalachian red spruce.

CONCLUSIONS

Despite occupying a relatively small geographical area, red spruce in the southern Appalachians display clinal variation in seed mass and bud set phenology related to climate, along with significant variation in filled seed and seedling germination. Faced with a warming climate, an important question is whether red spruce populations in the southern Appalachians have the capacity to respond to rapid changes in climate, as there are no natural migration routes in the present warming scenario that would permit a large-scale northward range shift. However, red spruce currently exists over a wide elevational range in the region, from roughly 900 to 2000 m, indicating that there are opportunities for taking advantage of naturally occurring genetic variation for restoration, depending on micro-climate and selection of appropriate stock. Our results support locally adaptive clines in seed and bud set traits, but the extent to which red spruce shows fine-scale adaptive genetic variation in other traits needs further exploration. New studies to quantify the extent of genetic variation in additional climate-adaptive traits across local environmental gradients in the southern Appalachians are imperative to better inform restorative efforts and decisions regarding appropriate seed sources to plant in a changing climate. Ideally, this would include reciprocal transplant experiments of high and low elevation seed sources into each respective environment to better understand local adaptation in physiological traits such as carbon allocation, photosynthesis, water use efficiency, and tolerance of temperature extremes. It may also be that the portfolio of genetic diversity and climate adaptation in the southern Appalachian red spruce will prove useful in maintaining resilient populations further north or at higher elevations, representing a form of assisted migration.

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