Genetic Variation in Needle Epicuticular Wax Characteristics in *Pinus Pinceana* Seedlings

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Abstract

Seedlings from each of 12 *Pinus pinceana* populations from throughout the species' range in Mexico were evaluated in a common-garden test to (1) determine the level of genetic variation and genetic structure of epicuticular needle wax quantity, (2) examine differences in wax chemical composition, and (3) seek evidence for an adaptive response in wax composition and quantity across environmental and geographic gradients. Regions and populations within regions showed high variation (38.2% and 10.5%, respectively, of the total variation) in wax quantity. Epicuticular wax recovered from primary needles of *P. pinceana* comprised eight classes. Secondary alcohols (71.7%) were the major homologs identified by gas chromatography. Seedlings from the northern region were separated based on wax composition from seedlings from the central and southern regions by canonical discriminant analysis. A strong differentiation among regions ($Q_{STR} = 0.571$) and populations within regions ($Q_{STP(R)} = 0.384$) was observed for wax quantity. Data on wax quantity and chemical composition indicate that physicochemical characteristics of epicuticular wax may show adaptation of *P. pinceana* to local environments.

Key words: environmental adaptation, epicuticular wax characteristics, genetic variation, physicochemical characteristics, *Pinus pinceana*, selection.

Introduction

Pinus pinceana Gordon is a piñon pine occurring in small, isolated populations in three regions along the Sierra Madre Oriental of Mexico, where annual precipitation ranges from about 360 to 800 mm (Perry, 1991). Despite its rarity, the species is significant because it provides food and shelter for wildlife and people in semi-arid woodland ecosystems (Perry, 1991). Its edible seeds are collected for human consumption, and trees or branches are harvested for firewood and rural construction. It is sometimes called weeping piñon because of its unusual and attractive form and this, along with its variation in needle color and ability to withstand extreme drought, holds appeal for urban planting.

Genetic variation and population genetic structure of epicuticular wax (EW) traits have been little studied in conifers. Quantification of additive genetic variation in wax quantity and the identification of its role in the adaptation of *P. pinceana* to xeric locations may provide useful information to support the development of a conservation strategy. This study's objective was to determine: (1) the level of genetic variation and population genetic structure for wax quantity in *P. pinceana* and (2) the extent to which the variation is adaptive.

Materials and Methods

Seedlings from six half-sib families in each of 12 populations in the three regions of Mexico where *P. pinceana* occurs naturally were included in this study (*Table 1*). Rainfall average was 30% lower in the northern region than in the southern region (*Table 1*). Thirty seeds from each half-sib family were germinated following the procedure described by Ramirez-Herrera et al. (2008) and seedlings were planted in 3.8 x 21 cm Ray Leach containers (Stuewe & Sons, Inc., Corvallis, OR, USA), filled using a 2:1 peat:vermiculite mix.

Containers were arranged in a randomized block design with regions, populations, and families randomized in each of six blocks. Five seedlings per family were planted in row plots in each block. Seedlings in two blocks were planted 23 d after seedlings in the first four blocks. Seedlings were grown in a greenhouse under the following conditions: 22°C, 12-h photoperiod, 60% relative humidity; 18°C, 12-h night, 50% relative humidity. They were watered and fertilized with a 20-08-20 fertilizer solution twice weekly. After 1 year, the seedlings were transplanted to 15 x 15 x 20 cm propagating pots (ROPAK CAN-AM Ltd., Springhill, NS, Canada) filled using a 2:1 peat moss:perlite mix.

Epicuticular wax amount and chemical analysis

Seedlings from each of six families from each of the 12 populations were included in wax analysis. Each family was represented by eight to 19 seedlings. Twenty primary needles were removed from the stem of each seedling at the end of the first year of growth. Wax was extracted according to the procedure described by Turunen et al. (1997). Six samples of *P. pinceana* from each of the three regions were analyzed for wax chemical composition using previously published techniques (Turunen et al., 1997).

Statistical analysis

Wax quantity residual data were analyzed for fit to the normal distribution using UNIVARIATE/SAS procedure for Windows version 9.1 (SAS Institute Inc., 2002). Data were transformed to their arcsine (θ = arcsine;

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Table 1. – Geographic location of 12 populations of *P. pinceana* Gordon.

Population	State	Region	Latitude (North)	Longitude (West)	Altitude (m.a.s.l.) †	Rainfall‡ (mm)	Dry season‡ (months) ‡‡
SJ Carbonerillas	Zacatecas	Northern	24°28′15″	101° 26′58″	2300	486	8
Lomas Oregano	Zacatecas	Northern	24°30′14″	101° 27′45″	2305	494	8
Santa Elena	Coahuila	Northern	25°01′47″	101° 24′39″	2077	483	8
Palmas Altas	Coahuila	Northern	25°08′02″	101° 26′56″	2090	479	8
El Cinco	Coahuila	Northern	25°10′35″	101° 41′14″	2250	463	8
El Recreo	Coahuila	Northern	25°17′44″	101° 00′03″	2238	507	7
Matehualilla	San Luis Potosí	Central	22°42′34″	100° 27′49″	2020	511	7
La Trinidad	San Luis Potosí	Central	22°40′02″	100° 28′20″	1945	519	7
Maguey Verde	Queretaro	Southern	21°05′18″	99° 41′48″	2176	748	6
El Tepozan	Queretaro	Southern	20°54′23″	99° 39′28″	2188	778	6
El Arenalito	Hidalgo	Southern	20°39′41″	99° 02′49″	1882	597	6
San Cristobal	Hidalgo	Southern	20°37′43″	98° 58′41″	1915	660	6

† Meters above sea level; ‡ Annual rainfall in mm and dry season estimated using the Spline Model (REHFELDT, 2006); ‡‡ Number of months with rainfall less than 10% of the annual rainfall in the population El Cinco.

$\sqrt{p}$; p = proportion of wax quantity/dried foliage) to improve normality (SOKAL and ROHLF, 1981). Wax quantity was subjected to ANOVA using GLM-SAS/PC for Windows version 9.1 (SAS Institute Inc., 2002). The ANOVA was based on the following model:

$$Y_{ijklmn} = \mu + A_i + B_{j(i)} + R_k + A*R_{ik} + B*R_{kj(i)} + P_{l(k)} + A*P_{il(k)} + B*P_{jl(k)} + F_{m(kl)} + A*F_{im(kl)} + B*F_{jm(ikl)} + \varepsilon_{ijklmn} \qquad [1]$$

where Y_{ijklmn} is seedling n of family m from population l in region k in block j at age i; μ is the overall mean; A_i is the age effect in days; $B_{j(i)}$ is the block-within-age effect; R_k is the region effect; $A*R_{ik}$ is the age x region interaction effect; $B*R_{kj(i)}$ is the block x region-within-age interaction effect; $P_{l(k)}$ is the population-within-region effect; $A*P_{il(k)}$ is the age x population-within-region interaction effect; $B*P_{jl(k)}$ is the block and population-within-region-within-age interaction effect; $F_{m(kl)}$ is the family-within-population-within-region effect; $A*F_{im(kl)}$ is the age and family-within-population-within-region interaction effect; $B*F_{jm(ikl)}$ is the block x family-within population-within-region-within-age interaction effect; and ε_{ijklmn} is the within-plot sampling error.

Region and populations were considered fixed effects, whereas age, block, and family effects were considered random. Tukey's studentized range test was applied for comparisons of region and population means. The variance components were estimated using VARCOMP/SAS for Windows version 9.1 (SAS Institute Inc., 2002) using the REML method, which considered all effects in the model as random.

The proportion of the differentiation due to region (Q_{STR}) vs. population-within-region ($Q_{STP(R)}$) for wax quantity was estimated using the following formulas that were deduced based on the equation described by SPITZE (1993):

$$Q_{STR} = \frac{\sigma^2_{GB}}{\sigma^2_{GB} + 2\sigma^2_{GW}} = \frac{\sigma^2_r}{\sigma^2_r + \sigma^2_{p(r)} + 6\sigma^2_{f(rp)}} \qquad [2]$$

$$Q_{STP(R)} = \frac{\sigma^2_{GB}}{\sigma^2_{GB} + 2\sigma^2_{GW}} = \frac{\sigma^2_{p(r)}}{\sigma^2_{p(r)} + 6\sigma^2_{f(rp)}} \qquad [3]$$

where σ^2_{GB} and σ^2_{GW} are the genetic variation among and within populations, respectively (SPITZE, 1993). σ^2_r, $\sigma^2_{p(r)}$ and $\sigma^2_{f(rp)}$ are the variance among region, population-within-region, and family-within-population variance, respectively.

The SEs for degree of differentiation among regions and populations were estimated based on 500 bootstrap replications at the level of family using the RANUNI function and the POINT option/SAS for Windows version 9.1 (SAS Institute Inc., 2002).

Canonical discriminant analysis using the CANDISC/SAS procedure for Windows version 9.1 (SAS INSTITUTE INC., 2002) was applied to chemical composition data.

Results

Quantitative variation

There were significant ($P < 0.05$) differences in wax quantity among regions, populations, and families (*Table 2*). Tukey's studentized range test indicated that the wax quantity from the northern region differed from the central and southern regions (*Table 3*). Population means were clustered in five groups. The overall mean for wax quantity was 13.5 mg g⁻¹, and ranged from 11.2 to 15.7 mg g⁻¹ for central and northern regions, respectively, and among populations from 9.5 for El Arenalito in the south to 16.6 mg g⁻¹ for Palmas Altas, a northern population (*Table 3*). The family means ranged from 8.7 to 18.4 mg g⁻¹.

The variation in wax quantity among families within populations accounted for 3.37% of the total, and the variation among populations within regions and among regions was 10.50% and 38.20%, respectively, of the total variation (*Table 2*). The proportion of the total differentiation for wax quantity that was among regions (Q_{STR}) and populations within regions ($Q_{STP(R)}$) was 0.571 (SE = 0.003) and 0.384 (SE = 0.005), respectively.

Wax chemical composition

Epicuticular wax recovered from primary needles of *P. pinceana* comprised eight classes (*Table 4*). The mean

Table 2. – Variance components as percentage of total variance (σ_r^2) for wax quantity of *P. pinceana* seedlings.

Variance Component	Degree of Freedom	Variance components as percentage of $\sigma_T^2 = 0.000301$
σ_a^2	1	0.07ns†
$\sigma_{b(a)}^2$	4	0.40ns
σ_r^2	2	38.20**
σ_{ar}^2	2	0.29ns
$\sigma_{br(a)}^2$	8	0.86ns
$\sigma_{p(r)}^2$	9	10.50**
$\sigma_{ap(r)}^2$	9	0.00ns
$\sigma_{bp(ar)}^2$	36	1.10ns
$\sigma_{f(rp)}^2$	60	3.37*
$\sigma_{af(rp)}^2$	60	0.00ns
$\sigma_{bf(arp)}^2$	217	0.39ns
σ_w^2	660	44.80

† Note: * and ** = significant levels at $\alpha < 0.05$ and $\alpha < 0.01$, respectively; ns = not significant. Significant difference test from the ANOVA performed using GLM/SAS; where σ_w^2 is the within-plot variance; $\sigma_{bf(arp)}^2$ is the block by family-within-population within-region-within-age variance; $\sigma_{af(rp)}^2$ is the age by family-within-population-within-region variance; $\sigma_{f(rp)}^2$ is the family-within-population variance; $\sigma_{bp(ar)}^2$ is the block by population-within-region-within-age variance; $\sigma_{ap(r)}^2$ is the age by population-within-region variance; $\sigma_{p(r)}^2$ is the population-within-region variance; $\sigma_{br(a)}^2$ is the block by region-within-age variance; σ_{ar}^2 is the age by region variance; σ_r^2 is the region variance; $\sigma_{b(a)}^2$ is the block-within-age variance; σ_a^2 is the age variance.

percentage of secondary alcohols, the major homologs identified by gas chromatography (GC), was 71.7% and ranged from 67.4% for the southern region to 75.5% for the northern region (*Table 4*). Alkanes, alkyl esters, and estolides constituted 23.2% of the homologs recovered. Fatty acids, dehydroabietic acid, primary alcohols, and diols were recovered in small proportions in *P. pinceana* wax.

The first canonical discriminant variable for EW chemical composition was significantly different from zero and explained 95% of the total variation, but the second canonical discriminant variable was not significant. The canonical correlation between the first canonical discriminant variable and the chemical composition was 0.94. The standardized canonical coefficients for the first canonical discriminant variable ranged from –3.496 for secondary alcohols to 2.854 for alkyl esters (*Table 5*). The correlation coefficients between the first canonical discriminant variable and the original variables varied from –0.419 to 0.607 (*Table 5*). The magnitude of the

Table 3. – Region and population means for wax quantity (mg g^{-1}) of *P. pinceana* seedlings.

Populations	Wax quantity mg g^{-1}	Family means (mg g^{-1})	
		Minimum	Maximum
Northern region	15.7A†	12.6	18.4
SJ Carbonerillas	15.8ab	13.5	17.1
Lomas Oregano	14.0bc	12.6	16.9
Santa Elena	16.1a	15.4	16.6
Palmas Altas	16.6a	15.0	18.4
El Cinco	15.9a	14.7	17.5
El Recreo	15.6ab	14.9	16.8
Central region	11.2B	9.7	12.9
Matehualilla	10.9de	9.7	11.5
La Trinidad	11.4d	11.1	12.9
Southern region	11.3B	8.0	14.7
Maguey Verde	13.4c	11.8	14.7
El Tepozan	12.6cd	10.9	13.8
El Arenalito	9.5e	8.0	10.6
San Cristobal	9.8e	8.7	10.4

† Means with the same letter are not significantly different among regions (uppercase letters) and within regions (lowercase letters) according to Tukey's studentized range test ($P < 0.05$).

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Table 4. – Chemical composition (%) of epicuticular wax recovered from primary needles of *P. pinceana.*

Chemical compounds	Northern region	Central region	Southern region
Fatty acids	1.7±1.27†	3.2±0.67	1.7±0.39
Alkanes	4.2±1.45	3.6±1.16	4.1±1.65
Dehydroabietic acid	0.6±0.55	0.3±0.26	2.1±2.12
Secondary Alcohols	75.5±3.63	72.3±1.84	67.4±3.09
Primary Alcohols	0.6±0.26	1.4±0.60	0.8±0.41
Diols	0.2±0.21	1.3±0.32	1.5±0.08
Alkyl esters	5.5±1.03	8.2±0.94	9.6±1.02
Estolides	11.8±3.49	9.7±2.51	12.8±1.86

† Standard error.

Table 5. – Standardized canonical coefficients and correlation between chemical composition and their canonical discriminant variables (Can).

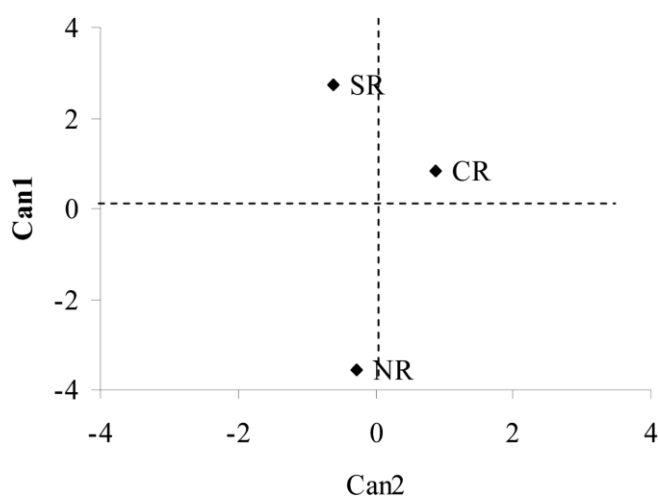| Loci	Can1		Can2	
	Canonical coefficient	Correlation coefficient	Canonical coefficient	Correlation coefficient
Fatty acids	0.665	-0.016	0.432	0.521
Alkanes	0.459	-0.198	-0.442	0.061
Dehydroabietic acid	-0.324	0.116	-0.143	-0.389
Secondary Alcohols	-3.496	-0.419	0.571	0.500
Primary Alcohols	-0.805	0.126	0.728	0.651
Diols	0.887	0.607	0.557	0.579
Alkyl esters	2.854	0.335	-0.888	0.327
Estolides	-0.983	-0.149	-0.293	-0.331

Figure 1. – Two canonical discriminant variables (Can) for chemical composition of epicuticular wax of *P. pinceana*. NR) northern region, CR) central region, and SR) southern region.

canonical coefficients and the correlation coefficients for the first canonical discriminant variable for chemical composition revealed that the differentiation among regions was mainly due to secondary alcohols, alkyl esters, and diols. The northern region was different from the other regions based on the first canonical discriminant variable for chemical composition (*Fig. 1*).

Discussion

In this study, *P. pinceana* populations in the northern region had the highest wax quantity and the highest proportion (75% vs. 67% in the southern region) of secondary alcohols. Together, these two physicochemical wax characteristics indicate an increased capacity for reflection of incident solar radiation (including UV-B) and potentially greater water-use efficiency (CAPE and PERCY, 1996; RICHARDSON et al., 2003). Northern populations likely experience more drought stress than central and southern populations as precipitation amounts are lower in the northern region (*Table 1*). It has been proposed that cuticular wax might improve drought tolerance in *P. taeda* L plants, with seedlings from xeric locations showing higher desiccation tolerance in needles than those from mesic locations (NEWTON et al., 1986). An indicator of moisture level in the *P. pinceana* stands may be associated with vegetation. For example, *Larrea tridentata* (DC) Cav. and *Prosopis glandulosa* Torr., two species that grow in arid regions where the annual rainfall is from 118 to 264 mm (BEATLEY, 1974; MONTAÑA et al., 1990), were found growing in association with *P. pinceana* only in the northern region of its natural range (MARES-ARREOLA, 2010).

Water stress is known to promote wax production in leaves (BAKER, 1982). Thus, many desert plants have a thick EW layer to prevent water loss. *Eryhea armata* S. Wats, e.g., has a glaucous wax bloom that allows this palm to adapt to dry conditions in the arid region of Baja California, Mexico (SCHMITT et al., 1993). DELUCIA and BERLYN (1984) found a strong relationship between leaf cuticle thickness, elevation, and the rate of cuticular water loss in *Abies balsamea*. POULOS and BERLYN (2007) examined needle morphology and phases of stomatal cuticular transpiration in relation to elevation in *Pinus cembroides*. Within their study area in Texas, elevation

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was positively correlated with rainfall. They found a linear relationship between all needle morphology traits and elevation, but cuticular transpiration was lowest at intermediate elevations, and the authors suggested that these individuals may be most adapted to variable conditions.

Pinus pinceana needle wax quantity exhibited high geographic variation, which may be associated with adaptation to local environments. High geographic variation in traits generally indicates adaptation to local environments (ZOBEL and TALBERT, 1984). This means that seedlings of trees that grow in favorable environments might not survive if they are planted in areas with extreme environmental conditions such as low temperatures and little precipitation, even when trees of the same species grow there. In the present study, variation in wax quantity among regions was three and a half times higher than variation among populations within regions, indicating strong differentiation among regions. Variation in wax quantity among regions and populations within regions was higher than variation in growth traits and branch number among regions and among populations within regions (RAMIREZ-HERRERA, 2007), suggesting that cuticular characteristics may be associated with adaptation of *P. pinceana* to local environmental conditions. Comparison of patterns of variation in wax quantity in *P. pinceana* with other conifers is difficult because no reports were found of studies covering such a large geographic area.

The values of Q_{STR} and $Q_{STP(R)}$ for wax quantity indicated that differentiation in *P. pinceana* was larger among regions than among populations within regions. However both Q_{STR} and $Q_{STP(R)}$ were higher for wax quantity than F_{STR} and $F_{STP(R)}$ estimates, based on allozymes (RAMIREZ-HERRERA, 2007). Based on the assumption that allozyme variability is neutral, this indicates that selection influenced differentiation in wax quantity among regions and populations within regions to a greater degree than did genetic drift. According to MERILÄ and CRNOKRAK (2001) when Q_{ST} is larger than F_{ST}, it can be interpreted to mean that selection favored individuals with particular phenotypes in different populations or regions in the present study. Both Q_{STR} and $Q_{STP(R)}$ in *P. pinceana* were higher for wax quantity than for other quantitative traits, including number of branches, height growth, and shoot biomass (RAMIREZ-HERRERA, 2007). There were no previously published reports of Q_{ST} estimates for wax quantity found in the literature. However, Q_{ST} reported for different traits in eight conifers averaged 0.379 (MCKAY and LATTA, 2002). Large Q_{ST} values have been reported for some traits in the *Pinus* genus. For instance, Q_{ST} values for height, stem form, and survival in *P. pinaster* Ait. were 0.791, 0.973, and 0.732, respectively (GONZALEZ-MARTINEZ et al., 2002). The Q_{ST} for timing of bud set in *P. sylvestris* was 0.86 (SAVOLAINEN et al., 2004).

The Q_{STR} and $Q_{STP(R)}$ for wax quantity in *P. pinceana* indicated that on average 57.1 and 38.3%, respectively, of the additive genetic variation were among regions and among populations within regions, and only 4.5% of additive genetic variation was within populations. This implies that wax quantity is or has been under strong selection as a result of local environmental conditions. In contrast, Q_{STR} and $Q_{STP(R)}$ values for several morphological traits in the species averaged 8.6% and 9.3%, respectively, indicating that 82.1% of the additive genetic variation was within populations for those traits (RAMIREZ-HERRERA, 2007). HAMRICK (2004) noted that typically <50% of total genetic variation for quantitative traits in conifers is among populations. The average among-population component of total additive genetic variation for quantitative traits in eight conifers was 37.9% (MCKAY and LATTA, 2002).

Although Q_{ST} values indicate a strong likelihood that adaptation underlies the observed geographic differences in wax quantity, the climatic drivers are less apparent. Mean wax quantity is positively correlated with both elevation ($r=0.73$; $P<0.01$) and latitude ($r=0.86$; $P<0.01$), implying that temperature may be a factor. BAKER (1982) and CAPE and PERCY (1993) stated that wax synthesis in leaves is influenced by temperature, irradiance, and light quality.

Secondary alcohols and estolides (GC-resolved) were the major chemical constituents of *P. pinceana* EW, as is usually the case for conifers (BAKER, 1982). For example, secondary alcohols and estolides represented 39.22% and 18.01%, respectively, in *P. ponderosa* wax although fatty acids, diols, alkyl esters, primary alcohols, and alkanes were also recovered in this species (BYTNEROWICZ et al., 1998). Secondary alcohols accounted for 40.2% of the chemical compounds in *P. silvestris* wax (TURUNEN et al., 1997).

The northern region was clearly distinguished from central and southern regions on the basis of wax chemical composition. On average, *P. pinceana* trees in the northern region likely experience higher drought stress than trees in the other regions because of the average rainfall and the distribution of it throughout the year (*Table 1*). Wax chemical composition, together with wax quantity, reveals that physicochemical characteristics of EW may be involved in adaptation of *P. pinceana* to semi-arid environments, as has been reported for other conifers. For example, EW chemical composition of *Araucaria araucana*, *Austrocedrus chilensis* [D.Don] Pic.-Ser. and Bizz, *Pilgerodendron uviferoum* [D.Don] Florin, *Juniperus communis* L. var. *saxitilis* Pall., and *J. communis* var. *montana* Aiton was associated with adaptation to the semi-arid conditions in which these species grow (RAFII and DODD, 1998; DODD and RAFII, 2000; DODD and POVEDA, 2003).

Conclusions and Recommendations

Pinus pinceana exhibited high levels of geographic variation for wax quantity and chemical composition. Most additive genetic variation for wax quantity was among regions and among populations within regions, revealing strong differentiation mainly due to selection. Wax quantity and chemical composition patterns indicate that physicochemical characteristics of epicuticular wax may be involved in adaptation of *P. pinceana* to arid environments.

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