Nut cold hardiness as a factor influencing the restoration of American chestnut in northern latitudes and high elevations

Thomas M. Saielli, Paul G. Schaberg, Gary J. Hawley, Joshua M. Halman, and Kendra M. Gurney

Abstract: American chestnut (*Castanea dentata* (Marsh.) Borkh.) was functionally removed as a forest tree by chestnut blight (caused by the fungal pathogen *Cryphonectria parasitica* (Murr.) Barr). Hybrid-backcross breeding between blight-resistant Chinese chestnut (*Castanea mollissima* Blume) and American chestnut is used to support species restoration. However, preliminary evidence suggests that backcross material may not have the cold hardiness needed for restoration in the northern portions of the species' range. The cold tolerance of nuts is of concern because reproductive tissues are particularly sensitive to freezing damage. We assessed nut cold tolerance for 16 American chestnut, four Chinese chestnut, and four red oak (*Quercus rubra* L.) (a native competitor) sources to better assess genetic variation in nut hardiness. We found that Chinese chestnut nuts were less cold tolerant than American chestnut and red oak nuts and that American chestnut sources from the south were less cold tolerant than sources from the north, with significant differences among sources within all regions. We also assessed how sources varied among temperature zones (sources separated by average winter temperature lows at source locations). Sources from the cold temperature zone were more cold tolerant and less variable in hardiness than sources from warm and moderate zones.

Résumé : Le châtaignier d'Amérique (*Castanea dentata* (Marsh.) Borkh.) a été fonctionnellement élimé comme essence forestière par la brûlure du châtaignier (causée par le champignon pathogène *Cryptonectria parasitica* (Murr.) Barr.). La méthode de rétrocroisement avec des hybrides entre le châtaignier chinois (*Castania mollissima* Blume) qui est résistant à la brûlure du châtaignier et le châtaignier d'Amérique est utilisée pour appuyer la restauration de l'espèce. Cependant, des données préliminaires indiquent que le matériel produit par rétrocroisement n'a peut-être pas la résistance au froid nécessaire pour la restauration dans la portion nord de l'aire de répartition de l'espèce. La tolérance au froid des noix est source d'inquiétude parce que les tissus reproducteurs sont particulièrement sensibles aux dommages causés par le gel. Nous avons évalué la tolérance au froid de 16 châtaigniers d'Amérique, quatre châtaigniers chinois et quatre chênes rouges (*Quercus rubra* L.) (un compétiteur indigène) pour mieux saisir la variation génétique de la résistance au froid des noix. Nous avons trouvé que les noix du châtaignier chinois étaient moins tolérants au froid que les noix du châtaignier d'Amérique et du chêne rouge et que les provenances méridionales de châtaignier d'Amérique étaient moins tolérantes au froid que les provenances septentrionales et qu'il y avait des différences significatives parmi les provenances dans toutes les régions. Nous avons également étudié comment les provenances varient entre les zones thermiques (provenances séparées selon les températures minimales moyennes en hiver à leur point d'origine). Les provenances de la zone thermique froide étaient plus tolérantes au froid et avaient une résistance au froid moins variable que les provenances des zones thermiques intermédiaire et chaude.

[Traduit par la Rédaction]

Introduction

American chestnut (*Castanea dentata* (Marsh.) Borkh.), a once dominant tree species in much of eastern North America, ranged from Georgia to Maine and west to the Ohio River Valley (Little 1977) where it represented up to 40% of the forest canopy (Keever 1953). American chestnut is an extremely fast-growing species (diameter growth as great as 2.5 cm/year) that can attain impressive proportions (e.g., heights of 37 m and diameters of 1.5–3 m, with a maximum diameter of 5 m observed in North Carolina, USA; Detwiler 1915; Buttrick 1925; Kuhlman 1978). American chestnut provided rot-resistant, straight-grained wood that was useful for construction, woodworking, furniture, railroad ties, telephone poles, mine timbers, and musical instruments (Anagnostakis 1987). Additionally, tannins from the wood and bark were integral to a large leather tanning industry (Fowler 1944), and the consistent mast of nuts from chestnut trees produced a nutritional mainstay for wildlife, domestic animals, and humans (Rice et al. 1980).

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T.M. Saielli, G.J. Hawley, and J.M. Halman. Rubenstein School of Environment and Natural Resources, University of Vermont, Burlington, VT 05405, USA.
P.G. Schaberg. US Department of Agriculture, Forest Service, Northern Research Station, Burlington, VT 05405, USA.
K.M. Gurney. The American Chestnut Foundation, South Burlington, VT 05403, USA.

Corresponding author: Paul G. Schaberg (e-mail: pschaberg@fs.fed.us).

American chestnut was functionally removed as an overstory tree approximately one century ago when chestnut blight (caused by the fungal pathogen *Cryphonectria parasitica* (Murr.) Barr) was accidentally introduced to the United States (Anagnostakis 1987). Over the years, there have been many approaches to overcome the impact of chestnut blight and restore American chestnut to its former ecological significance. These approaches included cultural methods such as the use of fungicides, tree surgery, or cutting and removing large swaths of chestnuts around infected sites (Beattie and Diller 1954). The propagation of hypovirulent forms of the pathogen has been effective in combating the disease in Europe but has been less effective in the United States (Elliston 1981; MacDonald and Fulbright 1991). Controlled breeding using native sources of American chestnut has occurred but has not produced trees with significant levels of blight resistance (Griffin 2000). One method that has shown significant promise of providing blight-resistant trees in the near term is the hybridization and backcrossing of American chestnut trees with blight-resistant Chinese chestnut (*Castanea mollissima* Blume) or Japanese chestnut (*Castanea crenata* Sieb. and Zuc.) (Griffin 2000; Hebard 2005). This method involves hybridizing Asian chestnuts with American chestnuts through controlled pollination and then successively backcrossing progeny selected for blight resistance with American chestnut to produce trees with approximately 94% American chestnut germplasm that are also blight-resistant (The American Chestnut Foundation 2010). Blight resistance is verified by assessing the degree of stem infection following challenge inoculations with the blight fungus.

In the northern extremes of American chestnut's former range, which includes Vermont, Maine, New Hampshire, and New York, USA, and southern portions of Ontario, Canada (Russell 1987), the restoration effort has an additional obstacle to overcome: the limited cold hardiness of American chestnut and backcross stock (Schaberg et al. 2009; Gurney et al. 2011). Indeed, field observations conducted by Gurney et al. (2011) indicated that some sources of American chestnut and backcross stock are vulnerable to winter injury and dieback of terminal shoots, which leads to a "bushy form" among seedlings. Some sources of American chestnut were less susceptible to winter injury (Gurney et al. 2011), indicating that the genetics of the parent trees are an important factor influencing hardiness. Furthermore, in preliminary experiments, Schaberg et al. (2009) found that American chestnut nuts are only cold tolerant to about 10 °C, a level that might not protect them from ambient winter temperature lows if they are not buried by animals or protected by snowpack. However, American chestnut sources may vary in nut cold tolerance by 5 °C or more (Schaberg et al. 2009), raising the possibility that genetic selection would likely increase nut cold tolerance to help bolster nut viability in colder climates. These variations in American chestnut cold tolerance are a likely result of regional adaptations to local climates that are influenced by latitude, elevation, proximity to large bodies of water, and other environmental factors.

The focus of this study was to better understand the magnitude and variation in nut cold tolerance among chestnut sources to potentially identify those with greater cold hardiness so that they might be incorporated into breeding programs and bolster nut survival in the coldest portions of its range. Nuts evaluated included four Chinese chestnut sources (essential for imparting blight resistance but speculated to be more vulnerable to freezing damage) and 16 American chestnut sources from the north, central, and southern portions of the species' range (current breeding relies heavily on germplasm from southern and central sources). Cold tolerance levels of four sources of northern red oak (*Quercus rubra* L.) acorns were measured to provide a comparison with hardiness levels of a sympatric competitor. In addition, nut cold tolerance estimates from this study were compared with measures of shoot winter injury for 12 of the American chestnut sources (data from a separate study: T.M. Saielli et al., in preparation) as a preliminary assessment of whether nut cold tolerance could be used as an indicator of shoot vulnerability to freezing damage.

Materials and methods

Nut sources

All American chestnut and Chinese chestnut nuts and red oak acorns used in this research were collected by members of our collaborative research group or were sent to us from locations throughout the eastern United States by staff and volunteers working with The American Chestnut Foundation. We used 120 nuts from 16 open-pollinated American chestnut sources, each representing one or more half-sib families located throughout the eastern United States, four Chinese chestnut sources, and four northern red oak sources (Table 1). American chestnut sources included six northern sources, five central sources, and five southern sources (Table 1). The four sources of Chinese chestnut were collected from trees growing in Vermont, Connecticut, Pennsylvania, and Missouri. However, because Chinese chestnut is indigenous to Asia, the Chinese chestnuts that we sampled were not expected to be locally adapted to the locations of parent trees but instead were used to explore generalized variability in the species. The four sources of red oak included three from Vermont and one from New Hampshire. Nuts were collected in the fall of 2008 and placed in individual zip-lock bags, covered in peat moss to prevent fungal growth, and kept slightly moist to prevent desiccation. Nuts were then refrigerated for at least 3 months at 3 °C to standardize environmental preconditioning prior to experimental freezing tests (Schaberg et al. 2009) and meet accepted stratification requirements (Baskin and Baskin 2001; Bonner and Karrfalt 2008).

Region and temperature indexes to estimate source cold tolerance

We hypothesized that cold tolerance in American chestnut is related to regional differences in winter temperatures associated with source origin. To broadly categorize these expected differences in hardiness, we first separated sources into northern, central, and southern regional groups. However, past research with a limited number of chestnut sources found that region based on latitude alone did not resolve broad differences in low-temperature acclimation for American chestnut nuts (Schaberg et al. 2009). Thus, for this study, we also created a new index of predicted low-temperature acclimation (Table 1) based on local air temperature data (NOAA National Climatic Data Center 2005) that was ad-

Table 1. Nut source codes, location information, region, longitude, latitude, elevation, mean minimum winter temperatures (December through February), and temperature zone for open-pollinated sources of American chestnut (*Castanea dentata*), red oak (*Quercus rubra*), and Chinese chestnut (*Castanea mollissima*) used in nut cold tolerance tests; each source represented one or more half-sib families.

Species	Source location (code, county, state)	Region	Latitude (north)	Longitude (west)	Elevation (m)	Mean minimum winter temperature (°C)†	Temperature zone
American chestnut	KY1, Metcalfe County, Kentucky	South	37°00′16″	85°37′34″	269	–2.50	Warm
	MD1, Montgomery County, Maryland	Central	38°57′53″	77°05′33″	100	–2.59	Warm
	MD2, Montgomery County, Maryland	Central	39°13′12″	77°25′27″	111	–2.59	Warm
	KY2, Metcalfe County, Kentucky	South	37°05′23″	85°18′24″	260	–3.12	Warm
	NC1, Jackson County, North Carolina	South	35°22′21″	82°47′29″	1387	–4.19	Warm
	NJ1, Monmouth County, New Jersey	Central	40°36′20″	73°07′10″	20	–5.05	Warm
	NY1, Westchester County, New York	North	41°19′41″	73°41′10″	94	–6.02	Moderate
	PA1, Franklin County, Pennsylvania	Central	39°59′38″	77°23′55″	600	–6.47	Moderate
	TN1, Fentress County, Tennessee	South	35°22′15″	84°06′31″	1173	–6.77	Moderate
	PA2, Mercer County, Pennsylvania	Central	41°20′58″	80°04′58″	384	–7.15	Moderate
	VA1, Smyth County, Virginia	South	36°49′40″	81°25′49″	1036	–7.62	Moderate
	NY2, Wyoming County, New York	North	42°37′44″	78°03′17″	417	–8.65	Moderate
	NH1, Hillsborough County, New Hampshire	North	42°49′07″	71°40′02″	85	–9.72	Cold
	ME2, Knox County, Maine	North	44°10′55″	69°08′09″	68	–10.81	Cold
	VT1, Chittenden County, Vermont	North	44°31′39″	73°12′11″	57	–11.12	Cold
	ME1, Piscataquis County, Maine	North	45°09′35″	69°04′58″	101	–12.12	Cold
Red oak	VT1, Grand Isle County, Vermont	North	44°38′27″	73°19′20″	108	–10.39	Cold
	VT2, Washington County, Vermont	North	44°18′46″	72°45′40″	484	–15.09	Cold
	VT3, Chittenden County, Vermont	North	44°33′12″	73°07′09″	383	–11.12	Cold
	NH, Strafford County, New Hampshire	North	43°24′35″	70°59′18″	602	–10.87	Cold
Chinese chestnut	CT, Tolland County, Connecticut	*	*	*	*	*	*
	VT, Chittenden County, Vermont	*	*	*	*	*	*
	MO, Boone County, Missouri	*	*	*	*	*	*
	PA, Huntingdon County, Pennsylvania	*	*	*	*	*	*

*Mother tree locations are listed, but region and other information were not available for native sources in China.

†Average minimum winter temperatures were calculated based on daily minimum air temperatures recorded over 10–30 years at local weather stations, averaged for each month and adjusted for elevation between weather stations and the source location using the environmental lapse rate 6.5 °C/1000 m.

justed for elevation (Fovell 2004). Mean minimum winter temperatures were calculated based on daily minimum temperatures recorded over 10–30 years at local weather stations, averaged for December, January, and February, and adjusted for elevation between weather stations and the source location using the environmental adiabatic lapse rate of 6.5 °C/1000 m. The range of winter low temperatures that these sources were exposed to was –2.5 through –12.1 °C; temperature zones were separated into nonoverlapping temperature ranges and operationally defined as warm (–1.0 through –5.0 °C), moderate (between –5.0 and –9.0 °C), and cold (colder than –9.0 °C).

Nut cold tolerance

Cold tolerance was quantified by measuring the electrolyte leakage from nuts following a series of controlled freezing tests. Assessing electrolyte leakage using an electrical conductivity probe has long been used as a quantitative assay for seed viability (e.g., Presley 1958; McDonald and Wilson 1979; Hopper and Hinton 1987; Wang et al. 2004; Schaberg et al. 2009). We assayed five nuts from each of the 24 sources. Nuts were washed in distilled water; pericarps, seed coats, and radicles were removed and the remaining cotyledons chopped into 5.0 mm cubes and then placed (one each) into individual compartments within 64-cell styrene trays. Past work has shown that cold tolerance levels derived from the experimental freezing of cotyledon material are indistinguishable from those calculated from germination tests following the freezing of whole nuts (Schaberg et al. 2009). Two cells per tray included one 5.0 mm cube from each nut, and duplicates within each tray were used to calculate mean electrical conductivity values used in curve-fitting analyses. We added 0.5 mL of 0.1% (*v/v*) Triton X-100 solution containing a bacterial ice nucleator (Snomax; Johnson Controls Inc., Centennial, Colorado) to each sample cell before freezing.

Freezing stress was imposed using well-established methods (Schaberg et al. 2008, 2009). We used 15 test temperatures ranging from 4 °C (no freezing stress) to –40 °C (inducing complete mortality). Temperatures were lowered at 2 °C/h increments and held for 30 min at each test temperature. After the specified test temperatures were reached, individual trays were removed from the freezer and allowed to slowly thaw in a walk-in cooler. Once thawed, an additional 2.0 mL of Triton X solution was added to bring the total solution per sample to 2.5 mL, and trays were then transferred to a high-humidity chamber and mechanically shaken for 8 h.

Initial conductivities of samples were measured using a multielectrode instrument (Wavefront Technology, Ann Arbor, Michigan). Samples were then dried at 45 °C for 48–72 h, reconstituted with 2.5 mL of Triton X solution, and then final conductivities measured. Relative electrolyte leakage (REL), a measure of cell injury calculated as the proportion of initial to final conductivity, was used to estimate tissue cold tolerance measured as T_m, an estimate of LT_{50} (temperature at 50% cell mortality) (Strimbeck et al. 2008). JMP statistical software (SAS Institute Inc., Cary, North Carolina) was used to calculate nonlinear curve fitting using the following equation (Anderson et al. 1988):

$$Y_T = Y_{\min} + \frac{Y_{\max} - Y_{\min}}{1 + e^{k(T_m - T)}}$$

where Y_T is the REL value at temperature T, $Y_{\min}$ is the asymptotic REL value in uninjured tissue, $Y_{\max}$ is the asymptotic REL value at maximum freezing stress, k describes the steepness of the REL response to freezing stress, T is the temperature in degrees Celsius, and T_m is the midpoint value of the REL sigmoid curve.

Statistical analyses

Analyses of variance (ANOVAs) were employed to test for differences in nut cold tolerance data (T_m) using JMP statistical software. One ANOVA tested for differences in cold tolerance attributable to "species" (all sources of American chestnut, Chinese chestnut, and northern red oak from Table 1 were utilized). For American chestnut data, nested ANOVA models tested for the influence of "region" (southern, central, and northern regions) and "source within region" as well as "temperature zone" (warm, moderate, and cold temperature zones) and "source within temperature zone" on nut cold tolerance thresholds. For Chinese chestnut and red oak data, ANOVAs tested for the influence of "source" on nut cold tolerance thresholds. The Tukey–Kramer HSD test was used to identify significant differences among means. The assumptions of homoscedasticity were met for each data set and no transformations were necessary. For all analyses, differences among means were considered statistically significant if $P \leq 0.05$.

Results and discussion

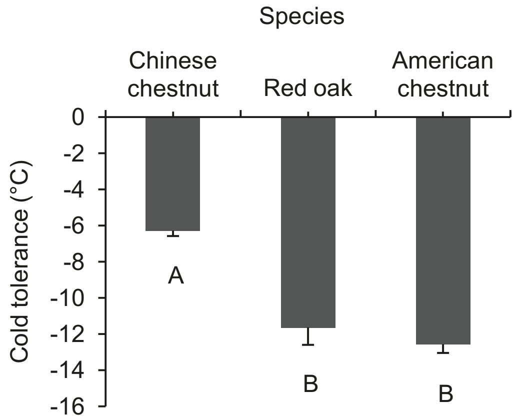
Species differences

Significant differences in nut cold tolerance were evident among the species (Fig. 1). Chinese chestnut nuts were significantly less cold tolerant than American chestnut nuts and red oak acorns, which did not differ significantly in hardiness. The mean cold tolerance of Chinese chestnut nuts was approximately –6.3 °C, whereas cold tolerance means for American chestnut nuts and red oak acorns were about –12.6 and –11.6 °C, respectively, collectively nearly 6 °C more cold tolerant than Chinese chestnut. These findings correspond to earlier studies that suggested that Chinese chestnut nuts have limited cold tolerance relative to American chestnut nuts (Jones et al. 1980; Schaberg et al. 2009; Gurney et al. 2011). Jones et al. (1980) found that Chinese chestnut shoots were susceptible to freezing injury at approximately –20 °C, whereas Gurney et al. (2011) found that the shoots of American chestnut and backcross stock were hardy to about –35 and –32 °C, respectively. Our nut cold tolerance data indicate that this same pattern of relative hardiness exists for Chinese and American chestnut nuts, although nuts were considerably more vulnerable to freezing damage than shoots. The mean nut hardiness for American chestnut (–12.6 °C) was about 2 °C more cold tolerant than levels previously published for the species (Schaberg et al. 2009). However, the species mean reported here included data from many more sources and specifically targeted the inclusion of northern and high-elevation sources presumed to be more cold tolerant.

Regional differences among American chestnut sources

Significant differences in nut cold tolerance were detected between the southern and the northern sources, with mean cold tolerance levels of approximately –8.9 °C for southern sources and –14.2 °C for northern sources (Fig. 2). Mean cold tolerance levels for nuts from central sources were intermediate to, and statistically indistinguishable from, those of southern and northern sources. The greater cold tolerance of northern sources is consistent with the existence of residual adaptation of populations to broad climate patterns (e.g., winter temperature lows) that a previous study, which examined only seven sources, was unable to detect (Schaberg et al. 2009). However, the considerable variation among sources within region (Fig. 2) suggests that factors other than gross latitudinal grouping affected source hardiness.

Fig. 1. Mean (±SE) nut cold tolerance estimated as T_m (estimate of temperature at 50% cell mortality) for Chinese chestnut (*Castanea mollissima*), red oak (*Quercus rubra*), and American chestnut (*Castanea dentata*). Means with different letters are significantly different based on Tukey HSD tests ($P < 0.0001$).



Not only were differences among sources within region common (existing for all regional groups), the range of cold tolerance between the least and most tolerant sources within each region was remarkably large and consistent, approximately 8 °C. Considerable within-region variability in nut cold tolerance may reflect the influence of smaller scale, localized site factors that influence cold tolerance physiology and adaptation. For example, site-specific environmental conditions could modify tree nutrition (e.g., nitrogen and calcium levels) and carbon metabolism (e.g., influencing the buildup of cryoprotective sugars) that can independently modulate cold tolerance physiology that supports nut cold hardiness (Schaberg et al. 2009). In addition, environmental gradients, such as elevation and proximity of populations to large bodies of water, that exist at more moderate landscape scales may be just as important as latitude in determining the severity of weather conditions that a nut source is exposed to. Indeed, considering that elevational differences within a region were sometimes greater than 1000 m (Table 1), it would be surprising if variations in cold tolerance among sources were not found within regional groups, indicating the need to assess localized climate variations in a way that is more comprehensive than region alone, such as we attempted by applying the concept of temperature zone.

Temperature zone differences among American chestnut sources

Significant differences in nut cold tolerance were also detected among temperature zones. Nuts from sources originating in the cold temperature zone were significantly more cold tolerant than nuts from sources from either the warm or moderate temperature zone (Fig. 3). Mean cold tolerance was –16.0 °C for nuts from the cold temperature zone, whereas nuts from the warm and moderate temperature zones were hardy to –10.3 and –12.5 °C, respectively, and were not statistically different from one another. Because the categorization of sources by temperature zone better isolated the hardiest sources than did the regional categorization (where northern sources were indistinguishable from central sources in hardiness), the temperature zone classification appears to be a better screen for identifying locations that harbor cold-tolerant populations. This possibility is supported by the low variability among sources within the cold temperature zone, which contrasted with the high source-to-source variability seen in the other temperature zones (Fig. 3).

The consistently high cold tolerance of nuts from the cold temperature zone may reflect more uniform selection for greater hardiness in areas where the influence of higher latitude and greater elevation and minimal moderating influences of water bodies all converged to create the lowest winter temperatures within the American chestnut's native range. Greater hardiness of nuts from the cold temperature zone may be best explained as a broad adaptation to cold within an area where it is a routine stress and selection pressure. Previous research by Howe et al. (2003) indicated that regional genetic adaptations are associated with differences in a species' cold tolerance.

The uniform hardiness of nuts from the cold temperature zone highlights the possibility that despite the dramatic reduction in reproductively mature sources of American chestnut following the introduction of chestnut blight, small breeding populations of residual chestnuts can still contain locally adapted traits (like greater cold hardiness), which can foster species restoration at marginal sites including the limits of the species' original range. Evidence of this apparent continued local adaptation underscores the need to include many sources of genetic variation within the context of regional breeding efforts.

The consistent hardiness of cold temperature zone sources may also simplify the identification and introduction of genes for greater hardiness into existing backcross breeding programs to bolster the hardiness levels of stock while also fostering blight resistance. However, because increased resource allocation toward elevated hardiness may come at the cost of reduced growth potential (T.M. Saielli et al., in preparation), breeding for greater cold tolerance must be balanced with considerations of other adaptations that promote long-term health, productivity, and the many ecosystem services that American chestnuts have historically provided. American chestnut is an example of a species that competes well with sympatric species in terms of rapid growth (Paillet and Rutter 1989; Latham 1992; McEwan et al. 2005; Jacobs 2007), but in the north and at higher elevations, growth potential must be balanced with the ability to survive freezing stress.

Although our data indicate that the temperature zone index better accounts for variations in nut cold tolerance than did the regional model, variation within the warm and moderate temperature zones suggests that factors other than recent winter temperature lows influence current nut cold tolerance levels. This indicates that historic exposure to the cold is not the sole driver of cold tolerance in these temperature zones. It is also possible that environmental differences at specific sites of existing, reproducing trees (especially those affecting tree nutrition and carbon relations, factors that greatly influence tissue cold tolerance; Schaberg and DeHayes 2000), could have modified tree and nut physiology, thereby influencing cold tolerance beyond those levels predicted by winter temperatures alone. In addition, the transport and planting of nonnative nuts were also common prior to the massive dieoff associated with the blight epidemic (Anagnostakis 2007).

Fig. 2. Mean (±SE) nut cold tolerance estimated as T_m (estimate of temperature at 50% cell mortality) among pure American chestnut (*Castanea dentata*) from three regions. Means with different uppercase letters for region are significantly different based on Tukey HSD tests (P < 0.0001) and lowercase letters for source within region differences (P < 0.005). Refer to Table 1 for source information.

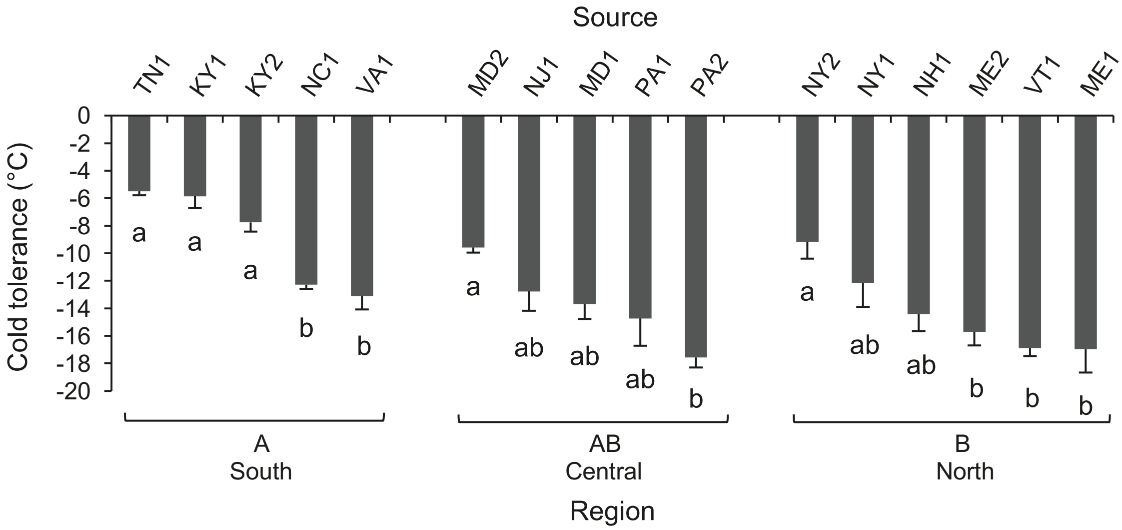
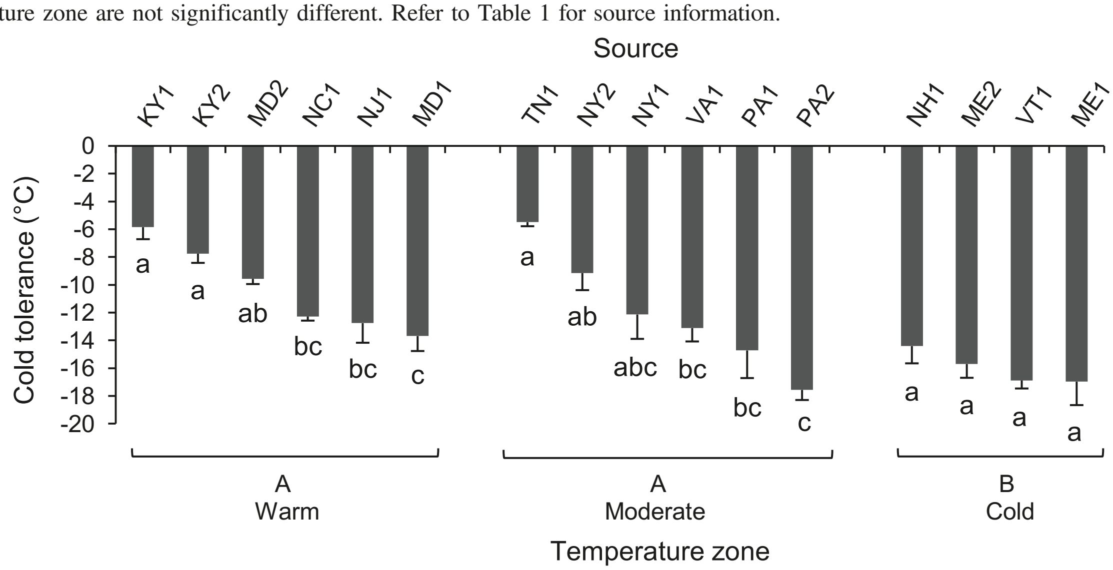


Fig. 3. Mean (±SE) nut cold tolerance estimated as T_m (estimate of temperature at 50% cell mortality) among pure American chestnut (*Castanea dentata*) sources from three temperature zones. Means with different uppercase letters for temperature zone and lowercase letters for sources within the warm and moderate temperature zones are significantly different based on Tukey HSD tests (P < 0.001); sources within the cold temperature zone are not significantly different. Refer to Table 1 for source information.



Thus, not all residual population enclaves are native, or uniquely adapted, to the locations where they are now found.

Differences among Chinese chestnut and red oak sources

There were significant differences in nut cold tolerance among the four sources of Chinese chestnut assessed (Fig. 4). Although we used the location of mother trees to distinguish Chinese chestnut sources, the specific genetic origins of these sources in China are unknown. Even though we cannot associate differences in hardiness among sources with geographic attributes, significant variation in nut cold tolerance among these sources indicates that, as was the case for American chestnut, "source" is critical to influencing the cold tolerance of Chinese chestnut. Thus, to increase the hardiness of nuts for hybrid and backcross progeny, it would be prudent to identify and use Chinese chestnut sources with high nut cold tolerance levels. However, in contrast with American chestnut sources, where temperature zone or regional indexes may expedite selection of sources with greater than average cold tolerance, there may be no surrogate indicator to help locate particularly hardy Chinese chestnut sources. Indeed, it is unclear if the Chinese chestnut stock readily available in the United States represents the peak hardiness of the species within its native range. It may be necessary to assess the cold tolerance of nuts from across China, Korea, and Taiwan to determine (*i*) if the limited hardiness that we measured here (Fig. 4) is characteristic of the species at large, (*ii*) if sources exist that show significantly elevated cold toler-

Fig. 4. Mean (±SE) nut cold tolerance estimated as T_m (estimate of temperature at 50% cell mortality) among Chinese chestnut (*Castanea mollissima*) sources. Means with different letters are significantly different based on Tukey HSD tests ($P < 0.0005$). Refer to Table 1 for source information.

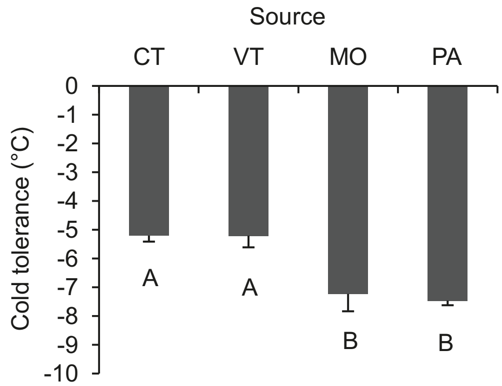


Fig. 5. Mean (±SE) nut cold tolerance estimated as T_m (estimate of temperature at 50% cell mortality) among red oak (*Quercus rubra*) sources. Means with different letters are significantly different based on Tukey HSD tests ($P < 0.001$). Refer to Table 1 for source information.

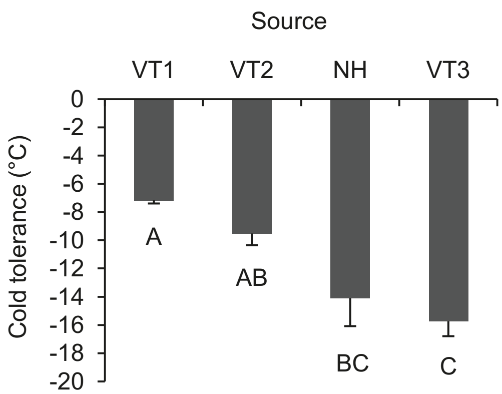
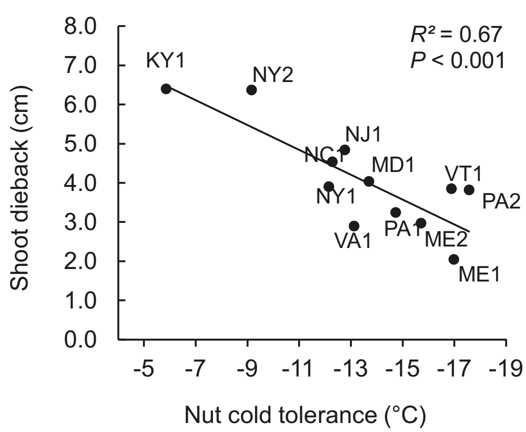


Fig. 6. Results of the linear regression between mean nut cold tolerance T_m (estimate of temperature at 50% cell mortality that was assessed in this study) and shoot dieback (assessed by T.M. Saielli et al., in preparation) for the 12 American chestnut (*Castanea dentata*) sources. Refer to Table 1 for source information.



ance, and (*iii*) if geographic or regional indexes are helpful in identifying the location of hardier sources within Chinese chestnut's native range.

There were also significant differences among the four red oak sources that we assessed from Vermont and New Hampshire (Fig. 5). Noted differences exemplify the high degree of variability that can exist among populations, even those within a similar region or temperature zone. Indeed, the range of cold tolerance levels for the four local New England sources of red oak (Fig. 5) was almost as great as the range for the 16 American chestnut sources that originated from a broad geographic distribution (Fig. 2).

Implications for breeding

Cold tolerance measurements based on REL data can produce slight overestimates of cold hardiness compared with thresholds of injury under field conditions because laboratory tests involve one exposure to a comparatively short freeze–thaw event, whereas the number and duration of freeze–thaw events are typically greater and produce more cumulative damage under field conditions (Schaberg and DeHayes 2000). Accordingly, although they allow for the precise comparison of relative hardiness levels among groups and individuals, the cold tolerance means that we present likely overestimated the ability of nuts to tolerate freezing stress in the field. However, ambient air temperatures in the northern portion of American chestnut's range can drop well below the hardiness levels that we report. Even if somewhat conservative, our estimates show a vulnerability to ambient air temperature lows and suggest that nut survival in the field relies on nut burial by rodents or the insulative buffer provided by snowpacks to protect nuts beyond levels of inherent hardiness. Nonetheless, our data showed significant and sometimes large differences in cold tolerance among sources (Figs. 1–5), indicating that genetic factors associated with species and source within species also influence the vulnerability of nuts to freezing damage that may alter nut viability and germinative success.

Backcross breeding programs aimed at producing blight-resistant chestnut rely on the hybridization of American and Chinese chestnut. It is unclear at this point what influence the small percentage of Chinese chestnut genes may have on resultant progeny, other than increased blight resistance. However, considering the results of our cold tolerance tests, there are several points to consider regarding the possible influence of Chinese chestnut and American chestnut germplasm on the cold tolerance of backcross nuts. First, due to the limited and low variability of nut cold tolerance, inclusion of Chinese chestnut germplasm could reduce the nut cold tolerance of backcross progeny. However, some Chinese chestnut sources may have greater cold tolerance than others. Thus, further analysis of differences in cold tolerance among Chinese chestnut sources is warranted. Although there is greater variation in cold tolerance among American chestnut sources, some have the capacity to be as cold tolerant as a native northern species: northern red oak. Thus, the species has retained the capacity to reproduce naturally in the cold fringes of its range. Considering the patterns of variation in nut hardiness that we identified, it is likely that there are sources of American and perhaps even Chinese chestnut that possess cold hardiness levels necessary for successful restoration of backcross chestnut in northern latitudes and higher elevations.

Temperature zone may be a reliable indicator of locations where sources are likely to be better adapted to low temperatures. In particular, sources from the cold temperature zone were more likely to be cold tolerant, whereas sources from the warm or moderate temperature zone were likely to be more variable in their hardiness levels and would require specific testing before use. Because sources within the cold temperature zone were consistently hardy, targeting sources within this zone would improve the probability of increased nut cold tolerance for progeny in backcross breeding programs.

In addition to its direct value in assessing overwintering viability, nut cold tolerance may be an important indicator of the hardiness of shoot tissues. As a preliminary assessment of this, we measured the linear association between nut cold tolerance (measured for this study) and shoot winter injury (assessed separately on plant material from the same sources; T. M. Saielli et al., in preparation) using regression analysis. Winter injury was identified after leaf-out as visible dieback (dark colored and sunken portions of stems) on terminal shoots and quantified by measuring the length (centimetres) of damaged material (shoot dieback). The significant linear regression between the mean nut cold tolerance (degrees Celsius) and shoot winter injury (centimetres) indicated that, in general, the greater the mean cold tolerance of nuts, the less shoot winter injury experienced by nut sources ($P < 0.001$, $R^2 = 0.67$) (Fig. 6). This relationship suggests that nut cold tolerance measurements (that can be obtained in weeks) may be a reasonable screening tool for identifying sources with greater shoot hardiness, a determination that typically requires advanced sapling growth and takes years to assess. Sources with maximum nut and shoot hardiness provide the greatest safeguard for successful nut survival and early vegetative growth in environments prone to low winter temperatures. If verified through additional testing, nut cold tolerance could be employed to rapidly assess the general hardiness of a range of available sources (American, Chinese, and backcross stock) and identify anomalous sources that differ from broad adaptive patterns (e.g., sources within warm and moderate temperature zones that showed elevated cold tolerance) (Fig. 3). Screening a variety of sources beyond those found in cold temperature zones would allow the inclusion of more sources, enhancing the genetic diversity of backcross stock while ultimately providing breeding programs with a wide range of sources that have the cold tolerance genes necessary for survival in colder climates.

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