

Decoupled evolution of foliar freezing resistance, temperature niche and morphological leaf traits in Chilean *Myrceugenia*

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Summary

1. Phylogenetic conservatism of tolerance to freezing temperatures has been cited to explain the tendency of plant lineages to grow in similar climates. However, there is little information about whether or not freezing resistance is conserved across phylogenies, and whether conservatism of physiological traits could explain conservatism of realized climatic niches. Here, we compared the phylogenetical lability of realized climatic niche, foliar freezing resistance and four morphological leaf traits that are generally considered to be adaptations to frost resistance in the Chilean species of *Myrceugenia*, which grows in a wide range of habitats.

2. We estimated the predicted niche occupancy profiles with respect to minimum temperature (minT) of all species. We measured foliar freezing resistance (using chlorophyll fluorescence), leaf size, leaf mass per area (LMA), stomatal and trichome densities of 10 individuals per species. Finally, we estimated phylogenetic signal, and we performed independent contrast analyses among all variables.

3. We found that both foliar freezing resistance and minT were subject to a significant phylogenetic signal, but the former had a stronger signal. We also detected a significant, but weak correlation between them ($r = 0.49$, $P_{\text{one tail}} = 0.04$). Morphological traits evolved independent of any phylogenetic effect.

4. *Synthesis.* Our results show that freezing resistance evolved in association with temperature niche, but with some delay that could result from phylogenetic inertia. Our results also show that morphological leaf traits are more labile than realized climatic niche and frost tolerance and that the formers probably evolved in association with microhabitat preferences.

Key-words: cold tolerance, functional traits, Mediterranean forest, phylogenetic niche conservatism, plant–climate interactions, realized climatic niche, temperate forest

Introduction

Understanding whether species retain ancestral ecological characteristics over evolutionary time-scales has become a major question in evolutionary biology (Harvey & Pagel 1991; Ricklefs & Latham 1992; Peterson, Soberon & Sanchez-Cordero 1999; Wiens & Graham 2005; Losos 2008; Pearman *et al.* 2008; Wiens *et al.* 2010; Crisp & Cook 2012). In the case of vascular plants, there is evidence suggesting that climatic distributions and other traits related with macrohabitat preferences are phylogenetically conserved

(Silvertown *et al.* 2006). Fossil records show that plant species often responded to Cenozoic climatic changes, tracking habitats and migrating to areas where the climate was similar to the one in which they evolved (Harvey & Pagel 1991; Ackerly 2004, 2009). Likewise, several comparative studies have shown that related species tend to grow in similar climates, latitudes or biomes (Qian & Ricklefs 2004; Crisp *et al.* 2009). These tendencies are expected to be shaped by the conservatism of physiological tolerances (Wiens & Graham 2005; Donoghue 2008). Particularly, the difficulty in evolving tolerance to freezing temperatures has been cited to explain the fact that many tropical groups did not expand to temperate regions during the Miocene when conditions

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became colder (Donoghue 2008) and also to explain latitudinal and altitudinal gradients in plant diversity (Hawkins, Rodriguez & Weller 2011). According to this hypothesis, Hawkins *et al.* (2014) showed that the minimum temperature experienced by North American tree species within their distribution range ('realized cold tolerance') evolved in a manner consistent with niche conservatism. However, there is little information about whether or not physiological freezing resistance is conserved across phylogenies and whether conservatism of physiological traits could explain conservatism of realized climatic niches (Crisp & Cook 2012).

Freezing is a major environmental stress, and limits plant growth at high altitudes and latitudes (Larcher & Bauer 1981). In general, freezing resistance tends to vary among plant species in accordance with their natural distributions (Larcher & Bauer 1981; Sakai & Larcher 1987; Araújo *et al.* 2013). However, some mismatches between distribution and frost resistance have been documented in comparative studies focusing on a single genus or a group of closely related species (Sakai 1970; Read, Hill & Hope 2010; but see Cavender-Bares 2007). For example, Read, Hill and Hope (2010) documented that foliar frost resistance of a New Guinea *Nothofagus* species was greater than expected for its climate and suggested that this anomaly might reflect the retention of traits evolved during Pleistocene glacial periods. Likewise, Sakai (1970) documented that tropical species of *Salix* suffer freezing damage at temperatures as low as their congeneric temperate species. These evolutionary trends suggest that freezing resistance might be subject to historical constraints, but this hypothesis has not been tested using an explicit phylogenetic framework.

In evergreen species, resistance of leaves to freezing temperatures is fundamental for plant survival. Foliar freezing resistance is associated with several morphological leaf traits that also increase water conservation and that can vary among plant species in association with climate (Reich *et al.* 2003). For example, reductions in leaf size, specific leaf area (SLA) and stomatal density, and increase in pubescence along rainfall and altitudinal gradients have been observed (Buot & Okitsu 1999; Fonseca *et al.* 2000; Wright, Reich & Westoby 2001; Knight & Ackerly 2003; Ordoñez *et al.* 2009; Sagaram, Lombardini & Grauke 2011). Although these trends suggest that morphological leaf traits are evolutionarily labile and are probably well adapted to environment, signals of phylogenetic conservatism (Ackerly 2004; Hinojosa *et al.* 2011) and a decoupling of morphological traits evolution with respect to habitat and climatic distributions have also been documented. For example, Ackerly, Schwilk and Webb (2006) showed that SLA evolved slower than climatic distributions in *Ceanothus*, whereas Savage and Cavender-Bares (2012) showed that morphological traits related to drought are more labile than habitat in *Salix*.

Myrceugenia is a genus endemic to southern South America, with two disjunct dispersal centres, one located in central Chile and the other in south-eastern Brazil (Landrum 1981a, b) that conforms with a separate lineage (Murillo *et al.* 2012). Twelve species of evergreen trees and shrubs of *Myrceugenia*

grow in continental Chile between 30° and 42°S, usually in wet habitats (e.g. swamps, banks of streams, temperate forest, fog-dependent forest). The majority of the species inhabit the Coastal Range at low and middle latitudes under a Mediterranean climate and are subject only to some episodic frost in winter. Other species grow in temperate forests and reach high altitudes with abundant snowfall in winter. Phylogenetic relationships among *Myrceugenia* species have been reconstructed using nuclear and cpDNA data (Murillo *et al.* 2012), and three clades within Chilean species were recognized. According to Murillo *et al.* (2012), northern species of the genus were placed in the basal clade suggesting that *Myrceugenia* evolved in warm climates and subsequently colonized southern areas with colder temperatures, and therefore, it is plausible that frost tolerance would be selected in this process.

In this study, we examined the evolution of temperature-climatic niche, foliar freezing resistance and morphological leaf traits in Chilean *Myrceugenia* species. First, we quantified the climate niches of each species, paying special attention to the minimum temperature of the coldest month (minT). Secondly, we measured several morphological leaf traits that are generally considered adaptations to frost resistance, including leaf area, leaf mass area, stomatal density and pubescence. Thirdly, we experimentally exposed leaves of all species collected in the field to increasingly cold temperatures, and then, we estimated the temperature producing 50% of damage (LT₅₀). Finally, we estimated phylogenetic signal (Pagel 1994), and we performed independent contrast analyses among all variables (Felsenstein 1985). In this way, we address the following questions: (i) Are minT-niche, foliar freezing resistance and morphological leaf traits subject to different levels of phylogenetic signal? (ii) Did foliar freezing resistance evolve in association with realized climatic niche, or Was the response of freezing resistance to environmental changes constrained by phylogenetic effects? (iii) Did foliar freezing resistance and morphological leaf traits evolve concertedly?

Materials and methods

CLIMATIC NICHE

We estimated the realized climatic niche of each *Myrceugenia* species from a total of 998 unique geo-referenced species records. We obtained the occurrences from national herbaria, Universidad de Concepción (CONC), Museo Nacional de Historia Natural (SGO), online data base (Global Biodiversity information facility, GBIF), Cooperative Taxonomic Resource for American Myrtaceae (CoTram) and our own field work. We modelled the climatic niche for each species employing the maximum entropy method (Phillips, Anderson & Schapire 2006) and using the 19 bioclimatic and topographic variables contained in the WorldClim climate data base (Hijmans *et al.* 2005) at one km resolution (30 s resolution). Background points were randomly chosen within the area enclosed by a minimum convex polygon comprising all records of the species. Occurrence data were partitioned 100 times into training and test data (80% and 20%, respectively) for model evaluation using the operating characteristic

curve (AUC). During these runs, the relative contribution of each variable to the final model was automatically determined by MAXENT. We used the *Myrceugenia* species probability (suitability) distributions derived from MAXENT to obtain their predicted niche occupancy (PNO) profiles with respect to minimum temperature of coldest month (minT). We also quantified the degree of niche overlap among taxa using Schoener's D, which ranges from zero (no overlap) to one (identity) (Schoener 1968; Warren, Glor & Turelli 2008; Evans *et al.* 2009). Finally, we estimated a weighted mean of minimum temperature for each species to be used in comparative analyses (hereafter, w-minT). All analyses were conducted in the R-package PHYLOC-LIM (Heibl 2011).

FOLIAR FREEZING RESISTANCE

We collected plant material of 12 Chilean *Myrceugenia* species (including two varieties of *M. ovata*) in August 2011 (mid-winter) from six sites (located between 33 and 41°S; Fig. 1) where 2 to 4 species co-occur naturally. The majority of species were sampled in one site, except for four species (*M. planipes*, *M. parvifolia*, *M. ovata* var. *nanophylla* and *M. chrysocarpa*) that were sampled in two sites. To assess whether differences between species in field conditions are the result of differences in acclimation conditions, we also measured frost resistance in seven species of *Myrceugenia* growing closely (less than 50 m apart) in the Botanical Garden of Viña del Mar (at 33.01°S, 71.31°W, Chile) under similar soil conditions. We collected 6-cm-long twigs from 10 individuals per species in each field site and from three individuals per species in the Botanical Garden. The twigs were wrapped in damp paper towels within polyethylene bags (to prevent further dehydration) and rapidly transported for freezing resistance analyses. We selected two mature sun-exposed leaves of the

previous growing season. Then, we measured the freezing resistance of leaves using chlorophyll fluorescence and following the protocol of Sierra-Almeida, Cavieres & Bravo (2010). Chlorophyll fluorescence has been widely used to estimate freezing tolerance (Cavender-Bares *et al.* 2005; Koehler, Center & Cavender-Bares 2012) and has been shown to correlate well with other estimators of freezing leaf damage, such as vital staining and leaf colour (Boorse, Ewers & Davis 1998). Two detached leaves per twig were introduced into separate, hermetically sealed plastic bags and incubated at one freezing temperature in a previously cooled cryostat (−4 to −20 °C, with intervals of 1 °C). Samples were kept at each temperature for 2 h to ensure homogeneous cooling. Then, the plastic bags were removed from the cryostat and left at 4 °C in the dark for 24 h. The control treatment consisted of samples placed in plastic bags and directly kept at 4 °C in the dark for 24 h (non-frozen samples). Different leaves and twigs were used for each treatment (two leaves per treatment, 17 treatments). We used an Efficiency Analyzer, Hansatech, Germany (Neuner & Buchner 1999), to determine the ratio of variable to maximum fluorescence (F_v/F_m) of dark-adapted leaves (Maxwell & Johnson 2000). Measures were conducted on each leaf before and after each freezing treatment. Then, we calculated the damage of each leaf as the percentage of photoinactivation ($PH_i = 1 - F_T/F_{MAX}$), where F_{MAX} is the F_v/F_m before treatment and F_T is the F_v/F_m after treatment. We plotted PH_i against the freezing treatment temperature to estimate the temperature producing 50% damage (LT_{50}). LT_{50} was estimated for each sampled individual separately.

MORPHOLOGICAL LEAF TRAITS

We collected 10 sun-exposed leaves per individual from the same individuals sampled for freezing resistance analyses. Leaves were scanned and dried in an oven at 60 °C for 2 days to obtain estimates of leaf area and leaf mass per area (LMA = dry mass/fresh leaf area; Cornelissen *et al.* 2003). Epidermis of a subset of five leaves per individual was dissociated in a commercial chloride solution and coloured with methylene blue (D'Ambrogio de Argüeso 1986). We estimated trichome and stomatal densities on two spots of 1 mm diameter located halfway along the length of the leaf and halfway between the midrib and leaf edge on the lower surfaces of the leaves. Mean values for each trait were estimated for each individual and each species.

PHYLOGENY AND COMPARATIVE ANALYSES

To evaluate phylogenetic signal, we used a time-calibrated phylogeny of *Myrceugenia* species previously published by Murillo (Murillo 2011; see also Murillo *et al.* 2012). *Myrceugenia* species that do not grow in continental Chile (from Juan Fernández Islands and south-eastern Brazil) were pruned out from the chronogram. Then, we estimated Pagel's lambda phylogeny-scaling parameter (Pagel 1994), which ranges from zero (when trait evolution is independent of phylogeny) to one (when trait evolution is strongly influenced by phylogeny and when species correlation is equal to the Brownian model expectation) (Pagel 1999). The lambda parameter was estimated for each trait separately (w-minT, LT_{50} , leaf size, LMA and stomatal and trichome densities) by maximum likelihood using the R-package Geiger (Harmon *et al.* 2009). The mean trait values for each species were used (estimated from two natural populations in the case of *M. planipes*, *M. parvifolia*, *M. ovata* var. *nanophylla* and *M. chrysocarpa*, and from one natural population for the remaining species). To determine whether the observed values differed significantly from 0.0 and from 1.0, we used the AIC criterion. To test whether freezing

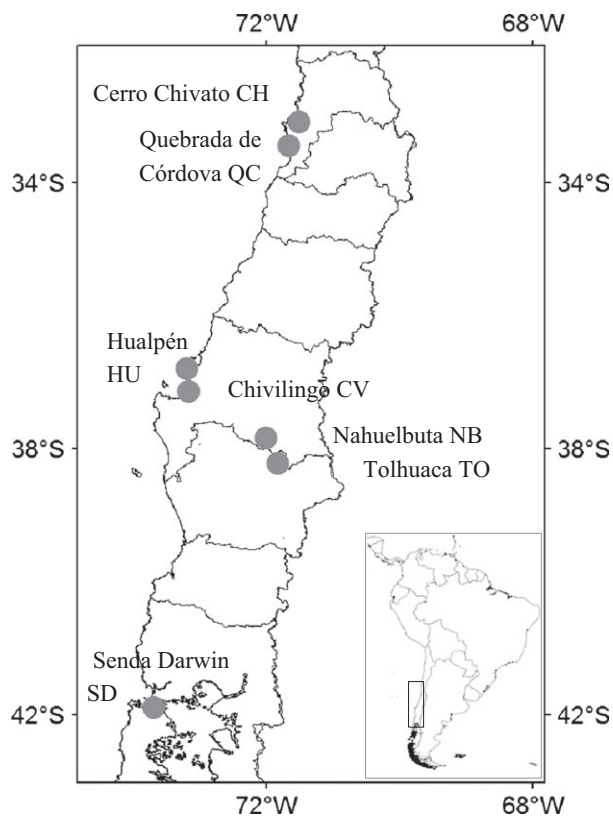


Fig. 1. Map of central Chile showing locations of study sites.

resistance, w-minT and leaf traits evolved concertedly in *Myrceugenia*, we performed phylogenetically independent contrasts for non-transformed traits (Felsenstein 1985). We tested the assumption of independence between contrasts and square of branch lengths, and given that no significant correlations were detected, we used contrast directly in statistical analyses. Then, we fitted multiple linear regressions (forced through the origin) to examine the relationships between leaf traits and LT_{50} , and between leaf traits and w-minT using the PDAP module (Midford, Garland & Maddison 2003) of the program Mesquite (Maddison & Maddison 2007). We also performed Pearson correlations between contrast for LT_{50} and w-minT to test whether freezing resistance evolved in correlation with winter temperature niche.

Results

TEMPERATURE NICHE

The PNO profiles in respect to minT (minimum temperature of coldest month) differed among the three major clades of Chilean *Myrceugenia* (hereafter, OVATA, PARVIFOLIA and RUFA clades, Fig. 2). In general, the species belonging to clade OVATA tended to occur in colder areas more than species of clade PARVIFOLIA and clade RUFA (Table 1 and Fig. 2). Overlap in PNO profiles within the PARVIFOLIA clade was high with all pairwise Schoener's *D* values above 0.8 (mean = 0.91, Table 2). Overlap was also high among species of clade OVATA (mean Schoener's *D* = 0.88), except for *M. colchaguensis*, which grows at higher temperatures than its relatives. Within clade RUFA, overlap was lower (mean Schoener's *D* = 0.68), being the most divergent species *M. rufa* and *M. correifolia*, which are the only species occurring in areas with minT greater than 7 °C (Table 1).

FOLIAR FREEZING RESISTANCE

Myrceugenia species growing in natural conditions showed a broad range of foliar freezing resistances with LT_{50} values ranging from -9.4 to -17.1 °C (Table 1). The most frost-resistant species were those that grow in the coldest areas, *M. chrysocarpa*, *M. ovata* var. *ovata* and *M. ovata* var. *nanophylla*, and their sister species *M. colchaguensis*, which grows at mid-temperatures (Fig. 2). Contrary to the expectation, the least frost-resistant species was *M. parvifolia*, which also grows at mid-temperatures. Interestingly, differ-

ences in LT_{50} between co-occurring species (from the same study site) were frequently greater than between related species from different sites. For example, individuals of *M. ovata* showed LT_{50} values 6 °C lower than individuals of *M. parvifolia* growing less than 100 m away in the SD (Chiloé), but only 1 °C lower than individuals of its sister species *M. colchaguensis* growing 1000 km northward in CH (Fig. 1).

Foliar freezing resistances varied between conspecific populations in two of the four species that were sampled in two sites. Individuals of *M. planipes* and *M. parvifolia* from HU showed higher LT_{50} values than their conspecific from SD, located 600 Km southward ($\Delta LT_{50} = 0.9$ °C for *M. planipes*; $\Delta LT_{50} = 1.2$ °C for *M. parvifolia*). However, these differences were lower than differences between individuals of *M. planipes* and *M. parvifolia* growing in sympatry (at SD: $\Delta LT_{50} = 2.7$ °C, and at HU: $\Delta LT_{50} = 3.0$ °C). No significant differences were detected among conspecific populations of *M. chrysocarpa* and *M. ovata* (Table 1).

Myrceugenia species growing in the Botanical Garden also differed in frost resistance. LT_{50} values recorded in the Botanical Garden were highly correlated with field measurements ($r^2 = 0.79$, $P > 0.01$; Fig. 4), although often field individuals were more frost resistant than their Botanical Garden conspecifics.

MORPHOLOGICAL LEAFTRAITS

Leaf traits varied significantly among *Myrceugenia* species (MANOVA: Pillai = 3.08, $F = 13.1$, $P < 0.001$). Leaf size showed a 75-fold range of variation, from less than 0.2 cm² in *M. rufa* to more than 15 cm² in *M. planipes* (Table 1). *Myrceugenia rufa* also showed the highest LMA (304 g m⁻²), which is five times greater than *M. parvifolia* (59 g m⁻²). Most species lack trichomes or have a low density, except for *M. rufa* and *M. correifolia*. All species are hypostomatic, but stomatal density was highly variable, with values ranging from 129 stomata mm⁻² in *M. obtusa* to 475 stomata mm⁻² in *M. ovata*.

PHYLOGENETICAL SIGNAL

The phylogenetic scaling parameter lambda did not differ significantly from 1.0 for w-minT ($\lambda = 0.73$) or LT_{50} ($\lambda = 1.0$),

Fig. 2. Predicted niche occupancy profiles with respect to the minimum temperature of the coldest month (minT) of Chilean *Myrceugenia* species mapped on the time-calibrated species tree produced by Bayesian analysis of nuclear and cpDNA data (modified from Murillo 2011). Y-axis, suitability or probability of species occurrence derived from Maxent.

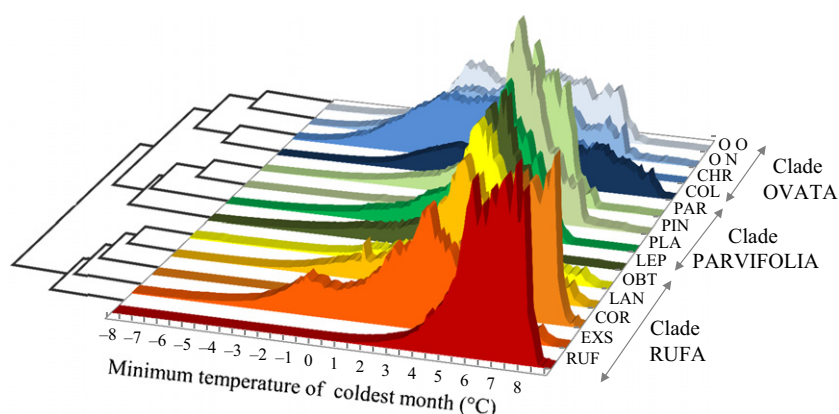


Table 1. Leaf traits, foliar freezing resistance and minimum temperature niche of *Myrceugenia* species. Site and locality from each species were sampled (Fig. 1); LS, leaf size; LMA, leaf mass per area; SD, stomatal density; TD, trichome density; LT₅₀, freezing temperature producing 50% foliar damage; w-minT, weighted mean of minimum temperatures and percentiles 10/90 derived from predicted niche occupancy profiles (Fig. 2); *ovata-nan.*, *Myrceugenia ovata* var. *nanophylla*; *ovata-ova.*, *Myrceugenia ovata* var. *ovata*. Values for leaf traits and LT₅₀ indicate the mean \pm SE obtained from 10 individuals. See site abbreviations in Fig. 1

Species		LS cm ²	LMA g m ⁻²	SD N mm ⁻²	TD N mm ⁻²	LT ₅₀ °C	w-minT °C
Clade RUFA							
<i>correifolia</i> (COR)	QC	7.61 \pm 1.18	230 \pm 13	439 \pm 42	27 \pm 12	-10.8 \pm 0.2	6.4 (4.3/7.9)
<i>exsucca</i> (EXS)	QC	8.00 \pm 1.34	158 \pm 21	349 \pm 10	1.8 \pm 1.9	-10.5 \pm 0.3	2.6 (-1.3/5.3)
<i>lanceolata</i> (LAN)	QC	3.02 \pm 0.51	172 \pm 4	428 \pm 24	0.4 \pm 0.8	-9.6 \pm 0.1	3.6 (0.5/6.3)
<i>obtusata</i> (OBT)	QC	1.15 \pm 0.18	159 \pm 14	129 \pm 10	0.9 \pm 1	-10.1 \pm 0.2	3.8 (1.4/5.9)
<i>rufa</i> (RUF)	CH	0.20 \pm 0.01	304 \pm 10	409 \pm 34	38 \pm 14	-9.7 \pm 0.3	6.7 (5.0/8.0)
Clade PARVIFOLIA							
<i>leptospermoides</i> (LEP)	CV	0.25 \pm 0.02	112 \pm 12	182 \pm 4	0.8 \pm 0.4	-10.8 \pm 0.4	3.3 (0.3/5.3)
<i>parvifolia</i> (PAR)	HU	1.14 \pm 0.02	59 \pm 1	200 \pm 2	1.0 \pm 0.4	-8.2 \pm 0.1	3.3 (0.6/5.2)
<i>parvifolia</i> (PAR)	SD	1.53 \pm 0.07	66 \pm 3	181 \pm 16	1.2 \pm 0.5	-9.4 \pm 0.1	3.3 (0.6/5.2)
<i>pinifolia</i> (PIN)	CV	0.32 \pm 0.03	129 \pm 17	282 \pm 16	1.1 \pm 2	-11.8 \pm 0.3	3.9 (2.2/5.4)
<i>planipes</i> (PLA)	HU	15.5 \pm 0.60	142 \pm 2	252 \pm 6	3.2 \pm 3.1	-11.2 \pm 0.3	3.0 (0.2/5.1)
<i>planipes</i> (PLA)	SD	7.58 \pm 0.40	169 \pm 8	215 \pm 4	5.2 \pm 4.7	-12.1 \pm 0.3	3.0 (0.2/5.1)
CLADE OVATA							
<i>chrysocarpa</i> (CHR)	NB	0.41 \pm 0.01	163 \pm 5	445 \pm 21	0.2 \pm 0.5	-17.1 \pm 0.3	0.1 (-3.5/4.8)
<i>chrysocarpa</i> (CHR)	TO	0.48 \pm 0.03	145 \pm 8	341 \pm 15	0.1 \pm 0.1	-17.6 \pm 0.6	0.1 (-3.5/4.8)
<i>colchaguensis</i> (COL)	CH	0.41 \pm 0.03	271 \pm 13	292 \pm 22	0 \pm 0	-15.1 \pm 0.2	3.1 (-0.5/6.7)
<i>ovata-nan.</i> (O_N)	NB	0.49 \pm 0.00	183 \pm 5	475 \pm 17	0.2 \pm 0.3	-17 \pm 0.3	0.9 (-2.7/4.8)
<i>ovata-nan.</i> (O_N)	TO	0.30 \pm 0.03	182 \pm 11	367 \pm 22	0.3 \pm 0.3	-17.0 \pm 0.3	0.9 (-2.7/4.8)
<i>ovata-ova.</i> (O_O)	SD	1.09 \pm 0.20	182 \pm 6	263 \pm 25	0.8 \pm 0.7	-15.9 \pm 0.3	1.4 (-1.9/5)

Table 2. Overlap among *Myrceugenia* species in predicted niche occupancy profiles with respect to minimum temperature of coldest month. Values correspond to Schoener's *D* statistic that ranges from zero (no overlap) to one (identity). See species abbreviations in Table 1

	Clade RUFA					Clade PARVIFOLIA				Clade OVATA		
	COR	RUF	OBT	LAN	EXS	PIN	LEP	PAR	PLA	O_O	O_N	CHR
RUF	0.88											
OBT	0.43	0.67										
LAN	0.4	0.66	0.98									
EXS	0.33	0.55	0.95	0.98								
PIN	0.36	0.56	0.86	0.75	0.70							
LEP	0.34	0.51	0.98	0.84	0.81	0.97						
PAR	0.32	0.5	0.86	0.82	0.81	0.97	0.93					
PLA	0.3	0.44	0.81	0.83	0.86	0.80	0.90	0.90				
O_O	0.26	0.18	0.57	0.69	0.80	0.54	0.66	0.66	0.72			
O_N	0.2	0.16	0.45	0.59	0.68	0.42	0.54	0.54	0.60	0.98		
CHR	0.34	0.27	0.73	0.83	0.89	0.67	0.81	0.8	0.84	0.96	0.98	
COL	0.73	0.67	0.93	0.97	0.97	0.89	0.92	0.91	0.92	0.91	0.90	0.55

indicating that both minT-niche and frost resistance are subject to strong phylogenetic signal (Table 3 and Fig. 3). In contrast, lambda values of morphological leaf traits were close to zero, suggesting that they evolved independently of any phylogenetic effects (Fig. 3). Phylogenetically independent contrast analyses detected a significant but weak correlation between LT₅₀ and w-minT (Pearson's correlation, $r = 0.49$, $P_{\text{one tail}} = 0.04$, Fig. 4), indicating that frost resistance and minT-niche evolved concertedly in Chilean *Myrceugenia*. Multiple regression of leaf traits on w-minT showed a significant effect of trichome density ($B = 0.07$, $P = 0.02$)

and a marginally significant effect of LMA ($B = 0.01$, $P = 0.06$), indicating that species found in warmer areas develop more dense and pubescent leaves. No significant association between leaf traits and LT₅₀ was detected (Multiple regression, all $P = \text{ns}$).

Discussion

We found that both foliar freezing resistance (LT₅₀) and minimum temperature (w-minT) were subject to a significant phylogenetic signal, but the former had a stronger signal

Table 3. Phylogenetic signal of minimum temperature niche, foliar freezing resistance and morphological traits. λ , phylogenetic scaling parameter of Pagel; AIC, Akaike information criterion for estimated lambda (AIC λ) and for testing models with $\lambda = 0$ and $\lambda = 1$; LT₅₀, freezing temperature producing 50% foliar damage; w-minT, weighted mean of minimum temperatures derived from predicted niche occupancy profiles (Fig. 2); LMA, leaf mass per area. Asterisks indicate the selected model with the lowest AIC

Trait	λ	AIC λ	AIC $\lambda = 0$	AIC $\lambda = 1$
w-minT	0.73	56.2	58.1	54.3*
LT ₅₀	1.00	62.0	70.4	59.9*
Leaf size	0.00	76.3*	76.3*	77.8
LMA	0.20	211.3*	213.4	236.7
Stomatal density	0.00	391.7*	391.7*	593.2
Trichome density	0.15	106.5	104.2*	105.8

(Table 3). We also detected a significant but weak correlation between these variables. Phylogenetic inertia might explain the low correlation among variables and the delay in the response of freezing resistance to climatic changes. *Myrceugenia colchaguensis*, for example, had a disjunct distribution with some populations located north of 33°S in the Coastal Range and others 500 km southward in lowland areas of the Andes Range. We sampled the northernmost population of this species, and we found that it retains the high frost resistance shown by its sister species (*M. ovata* and *M. chrysocarpa*), which reach high elevations with abundant snowfall. We hypothesize that range contraction during Pleistocene caused the current disjunct distribution of *M. colchaguensis*, and the unexpected high frost resistance resulted from phylogenetic inertia. On the other hand, *M. exsucca*, the most widespread species, showed low frost resistance resembling its sister

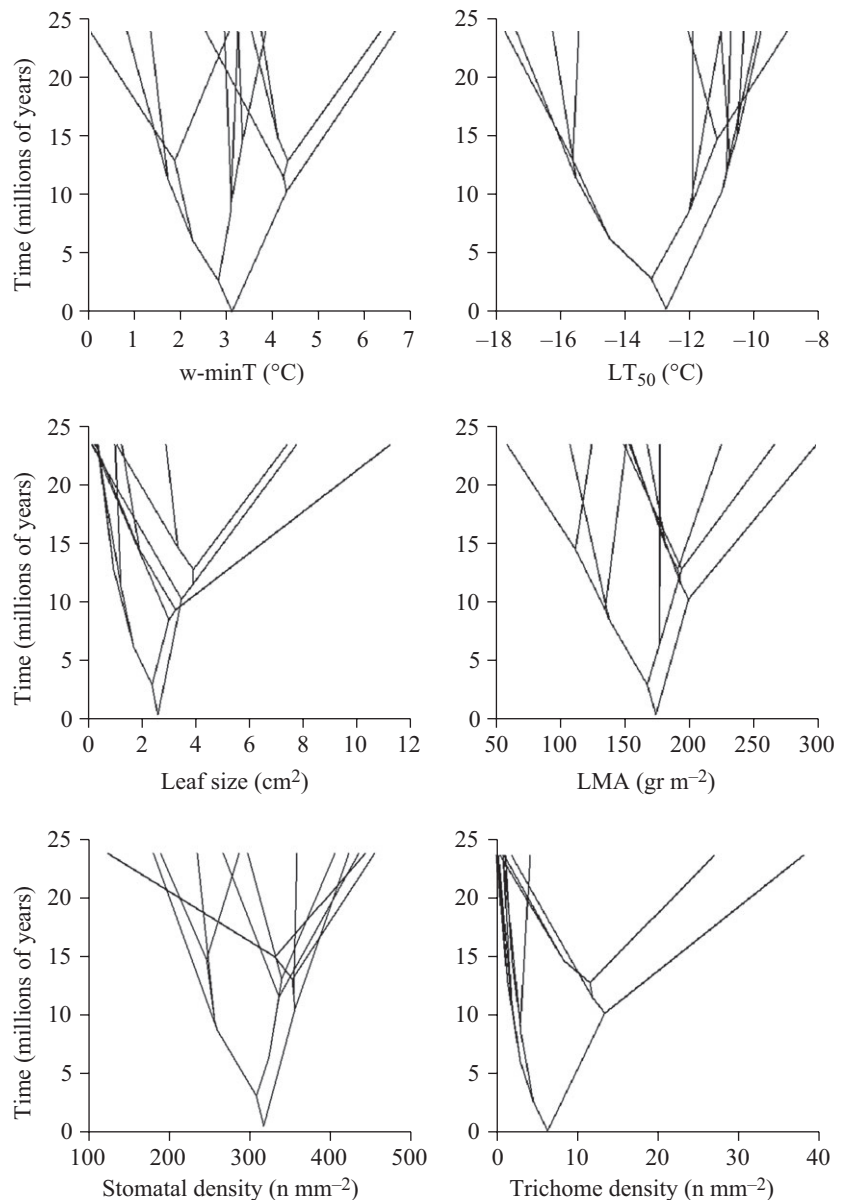


Fig. 3. Phylogenetic traitgrams for minimum temperature niche, foliar freezing resistance and leaf traits. Tips of the phylogenies are arrayed along the x-axis showing the species trait values. Y-axis, time of divergence from the common ancestor of *Myrceugenia*; LT₅₀, freezing temperature producing 50% of foliar damage; w-minT, weighted mean of minimum temperatures derived from predicted niche occupancy profiles (Fig. 2); LMA, leaf mass per area.

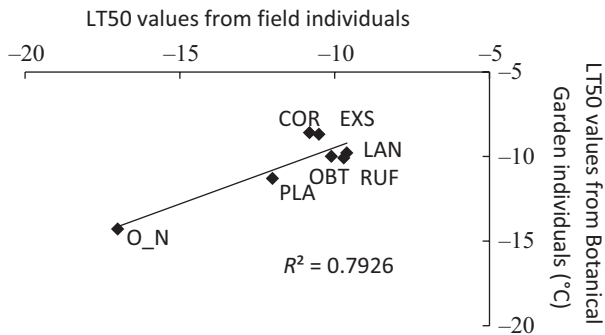


Fig. 4. Correlation between freezing resistance of individuals growing in natural conditions and conspecific individuals growing in the Botanical Garden. LT₅₀, freezing temperature producing 50% of foliar damage. See species abbreviations in Table 1.

species *M. correifolia*, which is restricted to north and lowland areas of the Coastal Range. *Myrceugenia exsucca* probably expanded its range southward during the Holocene following glacial retreat, and its low frost resistance may also result from niche conservatism. Further data from the southernmost populations of this species are necessary to confirm that this species conserves a low frost tolerance through all its distributional range.

Our results contrast with other comparative studies showing a high correlation between freezing resistance and climate, latitude or altitude (Sakai & Weiser 1973; Cunningham & Read 2006). However, most of these studies examined a pool of species from different lineages, ignoring phylogenetic effects. Anomalies in the relation between climate and frost tolerance have been documented in comparative studies focused on one genus or a group of closely related species (Sakai 1970; Read & Hill 1989; Read, Hill & Hope 2010; but see Cavender-Bares 2007), which might be the result of phylogenetic inertia and historical constraints. To our knowledge, phylogenetic signal on foliar freezing tolerance has not been previously tested using an explicit phylogenetic framework, but a significant influence of phylogeny on related biochemical traits (accumulation of dehydrin-like proteins and freezing behaviour) was documented for *Cornus* (Karlson *et al.* 2004).

It is important to emphasize that we sampled species from different sites. Therefore, we cannot discount that the observed differences between species can be the result of differences in acclimation conditions. Even so, three lines of evidence suggest that they have a genetic basis. First, observed differences in LT₅₀ between co-occurring species were frequently larger than between related species from distant sites. Secondly, variation within species seems to be lower than variation among species. We sampled two sites (SD and HU) separated by 600 km where *M. planipes* and *M. parvifolia* co-occur, and although we found that individuals from the northern site had higher LT₅₀ values than their conspecific from the southern site, these differences were lower than differences between individuals of *M. planipes* and *M. parvifolia* growing in sympatry. Thirdly, we found a high correlation between LT₅₀ values recorded in the field and in the

Botanical Garden, suggesting that differences in frost resistance among species are maintained even under similar climate and soil conditions in the cultivation and that these differences are consistent among sites. However, it is important to highlight that often field individuals were more frost resistant than their Botanical Garden conspecifics, which would be due in part to age differences (individuals in the Botanical Garden were much younger and shorter) or to some level of acclimation.

Phylogenetic conservatism of climatic niches and cold tolerances has been invoked to explain global gradients of biological diversity (i.e. Hawkins, Rodríguez & Weller 2011). For example, decreasing diversity of tree families towards lower latitudes has been explained by phylogenetic conservatism at the family level (Donoghue 2008). According to this hypothesis, cold tolerance would be the key innovation that allowed a reduced number of lineages (families) to persist at low latitudes when conditions became colder during the Eocene. As predicted by this hypothesis, Hawkins *et al.* (2014) showed that North American tree species from higher latitudes belong to younger families than trees species from lower latitudes. In the case of south-central Chile, a significant and positive correlation between diversity of tree species and annual temperature has been documented (Segovia *et al.* 2013). This pattern would also result from cold tolerance conservatism, but operating at lower taxonomic levels. Phylogenetic inertia would delay or hinder the colonization of new cold alpine environments originated with the Andean uplift and southern areas cooled after the Miocene Climatic Optimum (Hinojosa 2005). Our results suggest that cold tolerance tends to be conserved within major clades of *Myrceugenia* (which were originated between 15 to 17 millions of years; Murillo 2011), providing some support for this hypothesis.

We found that leaf morphological traits are more evolutionary labile than freezing resistance. These results agree with Silvertown *et al.* (2006), who characterized the niche at hierarchical scales (α , local; β , macrohabitat) and proposed that β -niche-related traits (such as climatic tolerances and latitudinal ranges) should be less labile than α -niche-related traits (such as those related with segregation across hydrological and light gradients). According to Silvertown *et al.* (2006), contrasting signals of α - and β -niche-related traits would allow for coexistence of closely related species. In our study, we measured leaf traits associated with drought tolerance, and their high lability would allow segregation of *Myrceugenia* sister species across hydrological gradients. Indeed, we observed several species of the RUFULA clade growing in sympatry at QC (Fig. 1) and other areas of central Chile with different microhabitat preferences. For example, *M. lanceolata* and *M. exsucca* were often found in waterlogged soils in creek beds, whereas *M. rufa* and *M. correifolia* their close relatives were usually found in drained soils of creek slopes. The species of the PARVIFOLA clade follow the same pattern at HU and CV sites, with *M. parvifolia* (HU) and *M. pinifolia* (CV) growing on creek beds, and *M. planipes* (HU) and *M. leptospermoides* (CV) growing few metres

uphill. In all the cases, the species from drained soils have leaf traits that decrease transpirational water loss: that is, the leaves of *M. rufa* and *M. correifolia* were more pubescent and had higher LMA than *M. lanceolata* and *M. exsucca*; *M. planipes* had higher LMA than *M. parvifolia*; and *M. piniifolia* had lower density of stomata than *M. piniifolia* at CV. Leaves that are more dense and rigid (High LMA) or have low stomata density have smaller transpiring surfaces (Poorter *et al.* 2009). Likewise, greater pubescence increases boundary layer resistance (Fahn 1986; Baldini *et al.* 1997).

We failed to detect an association between morphological leaf traits and frost resistance, but we found a significant and positive relationship between w-minT and both LMA and pubescence, suggesting that species growing in warmer areas develop denser and more pubescent leaves. It is well known that drought and cold stress can generate similar response in plants (Chaves, Maroco & Pereira 2003), and therefore, the observed relationships between temperature and leaf traits in *Myrceugenia* could be confused with response to precipitation. Indeed, in Chile, mean annual precipitation and mean annual temperature are highly and negatively correlated (Segovia *et al.* 2013).

Overall, our results show that freezing resistance evolved in association with temperature niche in *Myrceugenia*, but with some delay that could result from phylogenetic inertia. Morphological leaf traits are more evolutionary labile than freezing resistance traits, and the former probably evolved associated with microhabitat preferences.

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Data accessibility

Data for this study has been deposited in the Dryad repository (Pérez *et al.* 2014).

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