

Do 120,000 years of plant–pollinator interactions predict floral phenotype divergence in *Calceolaria polyrhiza*? A reconstruction using species distribution models

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Abstract Quaternary climatic changes impacted species' demography and distribution worldwide. Although response to climate change could have been modulated by mutualistic interactions with other species, studies exploring the dynamics of these interactions and their role facilitating species persistence during past climatic variations are scarce. In this work, we attempt to explore the spatial dynamic of *Calceolaria polyrhiza* and its oil-collecting bee pollinators during the last 120,000 years, identifying stable areas of persistence and statistically determining whether the distribution of pollinator-related floral ecotypes is associated with these shared areas of persistence. To do this, we used 395 presence records of the interacting species and constructed species palaeodistribution models. Additionally, we gathered phenotypic measures of the plant and used decision tree and multiple regression analyses to link the plant phenotypic divergence

with the distribution of stable areas. Our species distribution models suggest that past climatic changes affected the interaction between *C. polyrhiza* and both bee species in time and space. While the interaction between the plant and *C. caeruleus* predominated in the Andean-Patagonian forest and was relatively stable in space and time, that was not the case for the pollinator *C. cineraria* in the Patagonian steppe. This, along with our analyses of spatial phenotypic divergence, indicates that current floral phenotypes are the result of two historical different pollination regimes.

Keywords *Centris* · *Chalepogenus* · Climatic stable areas · Patagonia · Pleistocene · Specialized mutualism

As our research teaches, the bee groups in question, in view of their detailed adjustments apparently ab origine, were very closely linked to oil flowers. This emerges directly from: their partnership as their legitimate pollination symbionts, from the concordance of their geographic ranges, and the very close mutual relationships.

S. Vogel, 1974.

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Introduction

Numerous studies suggest that Quaternary climatic changes had a major impact on species demography and distribution, affecting the composition and structure of many communities worldwide (Hewitt 2000; Hickerson et al. 2010; Gavin et al. 2014). Such impacts have been relatively well studied in the Northern Hemisphere (e.g. Hewitt 1996; Taberlet et al. 1998; Alsos et al. 2005; Mraz et al. 2007; Waltari et al. 2007) and in some regions of the Southern Hemisphere (Beheregaray 2008; Sérsic et al. 2011; Fraser et al. 2012; Turchetto-Zolet et al. 2013; Prates et al. 2016).

However, while it is suspected that the response of organisms to climatic variation has been modulated by interspecific interactions (Davis et al. 1998; Bronstein et al. 2004; Thompson et al. 2005; Valiente-Banuet et al. 2006; Wisz et al. 2013), studies exploring the historical dynamics of these interactions are scarce. Thus, there seems to be limited information on the role played by ecological processes (e.g. interspecific interactions) on the persistence of species during past climatic changes (Thompson et al. 2005; Valiente-Banuet et al. 2006). Specifically, it is not fully understood whether or not mutualistic partners persisted, migrated, or expanded jointly in response to Pleistocene climate and landscape changes, and if these shared historical processes influence their phenotypic evolution (e.g. Espíndola et al. 2014; Triponez et al. 2015).

Oil-secreting flowers and oil-collecting bees represent one of the most specialized plant–pollinator systems among angiosperms (e.g. Vogel 1988; Johnson and Steiner 2000; Machado 2004; Sérsic 2004; Cosacov et al. 2008). Flowers offering fatty oils are found only in eleven families of flowering plants (Buchmann 1987; Neff and Simpson 2005; Renner and Schaefer 2010). Flowers from these plants are visited by highly specialized bees (Buchmann 1987; Cocucci et al. 2000, Machado 2004), which collect the nonvolatile floral oils and use them for brood provisioning and for lining their underground nests (Vogel 1974). Since the first discovery of this pollination reward in *Calceolaria*, several studies have investigated this specialized interaction between *Calceolaria* and its oil-collecting bees (Sérsic 2004; Cosacov 2010; Cosacov et al. 2009, 2012, 2014; Renner and Schaefer 2010; Martins et al. 2013; Murúa et al. 2014; Murúa and Espíndola 2015).

In Southern South America, one of these interactions has been described for *Calceolaria polyrhiza* Cav. (Calceolariaceae), a perennial herb endemic to Patagonia (Fig. 1). This species is exclusively pollinated by *Centris cineraria* (Apidae: Centridini; Simpson and Neff 1981; Roig-Alsina 1999, 2000) and *Chalepogenus caeruleus* (Apidae: Tapinotaspidini; Simpson and Neff 1981; Roig-Alsina 1999, 2000; Sérsic 2004; Cosacov et al. 2014). Because these two oil-collecting bee species greatly differ in body length and oil-collecting behaviour (Molau 1988; Roig-Alsina 1999, 2000; Sérsic 2004; Cosacov 2010), a recent study (Cosacov et al. 2014) suggested that they played a key role in shaping floral phenotype, promoting the differentiation of two spatially distinct floral ecotypes (i.e. short vs. long throat for *Chalepogenus* vs. *Centris* pollinated flowers, respectively; Fig. 1b, c).

Several aspects make *C. polyrhiza* an ideal system to explore to what extent the mutualistic species persisted or migrated jointly in response to past climate change, and to what extent these historical processes directed the development/evolution of floral phenotypes. First, it has been

shown that the demography and range of *C. polyrhiza* have been significantly affected by Pleistocene climatic changes (Cosacov et al. 2010; Sérsic et al. 2011). Second, this species has a predominantly xenogamous reproductive system (Cosacov 2010), indicating that cross-pollination is required and that pollinators may apply strong selection on floral traits. Third, it shows great floral phenotypic variation across its range, which has been shown to be associated with pollinator identity (Cosacov et al. 2012, 2014). Finally, historical effects would have likely influenced the adaptive evolution of this plant species (Maubecin et al. 2016).

One way to infer historical range variation among interacting species is through the use of species distribution models (SDMs). SDMs have been recently developed to project species distributions using a combination of species occurrences and environmental variables (Elith and Leathwick 2009). Projections of niche models of the species onto environmental reconstructions for different time periods can allow us to estimate changes in the range of species over time (e.g. since the Last Glacial Maximum; Espíndola et al. 2012). If used for interacting species, the methodology can provide information for the persistence (or not) of interacting species in shared areas during large periods of time. Thus, using this approach could inform our understanding of the dynamics of the interaction in space and time.

The aims of this study are: (1) to characterize the spatiotemporal dynamic of the ranges of each bee species (*Chalepogenus caeruleus* and *Centris cineraria*) and of the host plant *Calceolaria polyrhiza*; (2) to detect stable shared areas for *C. polyrhiza* and its bees; (3) to determine statistically whether the distribution of each floral ecotype (i.e. short vs. long throat) is associated with shared areas of long-lasting persistence of different pollinators (i.e. short throat with *C. caeruleus* and long throat with *C. cineraria*).

Materials and methods

Species occurrence data

Species occurrences were mainly obtained from field collections and complemented with data obtained from the literature (Roig-Alsina 1999, 2000), museum collections (AMNH, BBSL INHS), herbarium voucher specimens (IS) and global databases (GBIF, USDA-ARS) (see Online Resource 1). A total of 395 records were compiled (Fig. 1): 208 for *C. polyrhiza*, 108 for *C. cineraria* and 79 for *C. caeruleus*. Special attention was taken to use adequate numbers of occurrences that covered the entire ranges of the species.

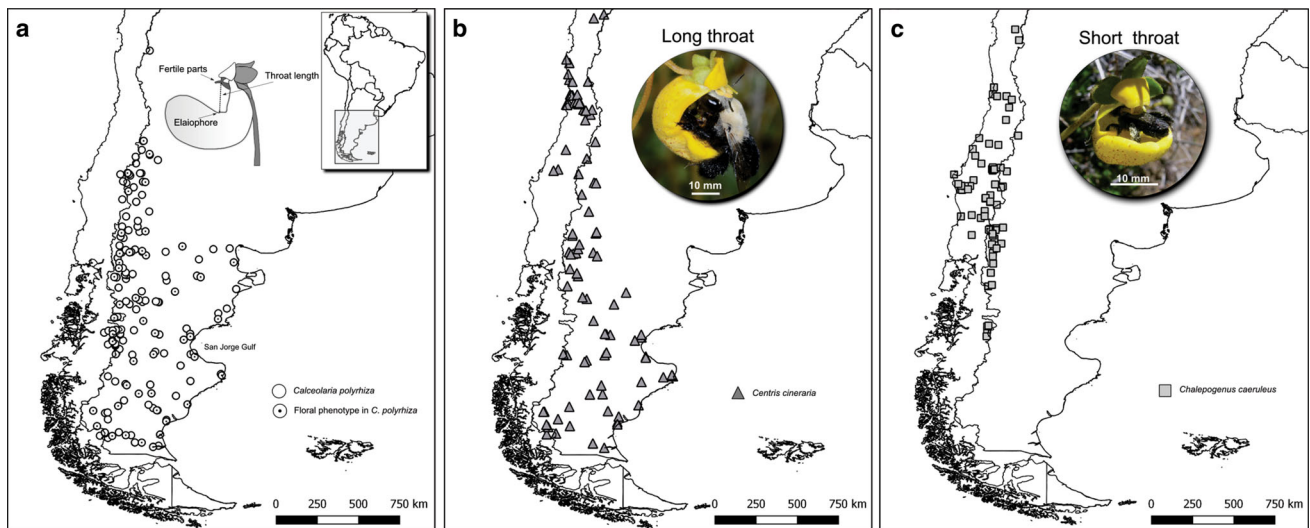


Fig. 1 Localities used in the distribution modelling analyses for the three species. The study area is shown in the *inset*. **a** *Calceolaria polyrhiza* localities and schematic diagram of a flower. **b** *Centris cineraria* localities and a picture showing the bee collecting oil in a

long throat floral morphotype. **c** *Chalepogenus caeruleus* localities and a picture showing the bee collecting oils in a short throat floral morphotype

Environmental data and climatic reconstructions

Current and past climatic reconstructions were downloaded from the WorldClim database (Hijmans et al. 2005; <http://www.worldclim.org>). Current conditions have been calculated for the 50-year period from 1950 to 2000, while past conditions corresponded to the Last Glacial Maximum (LGM, ~21 ka) and Last Interglacial period (LIG, ~120–140 ka; Otto-Bliesner et al. 2006). These two past periods correspond to two opposite climatic extremes during the Pleistocene (Rabassa 2008) and may represent key events in the recent evolutionary history of Patagonia. For the LGM, we used the Model for Interdisciplinary Research on Climate (MIROC). For each time period, we used nineteen bioclimatic variables representing annual trends, seasonality and extreme environmental conditions, at a spatial resolution of 2.5 arc-minutes (~5 km²) for the present and the LGM and at 30 arc-seconds resolution (~1 km²) for the LIG, as available in the WorldClim database (<http://worldclim.org/bioclim>).

Species distribution modelling

We ran the SDM for each species using the maximum entropy algorithm in MAXENT v3.3.3k (Phillips et al. 2006) and then projected it using QGIS v2.14 (QGIS Development Team 2016). The modelling training region for *C. polyrhiza* and *C. cineraria* was from 27°49.87'S to 51°56.97'S and from 72°34.37'W to 65°42.7'W, and for *C. caeruleus* from 28°24.37'S to 46°9.22'S and from 74°6.92'W to 70°14.15'W. To project the SDM, all bioclimatic layers were cropped to span from 21°38.5'S to

56°43.99'S and from 76°43.99'W to 53°1'W, covering a larger spatial range than the current Andean-Patagonian forest and Patagonian steppe (following Owens et al. 2013).

In MAXENT, we selected the autofeatures option, which allows for linear, quadratic, product, threshold and hinge features to describe relationships between occurrences and environmental conditions (Merow et al. 2013). Additionally, we used logistic outputs, regularization multiplier = 1, random test percentage = 25, convergence threshold = 0.00001 and maximum iterations = 1000. A total of 10 bootstrap model replicates were generated for each model. Model performance was evaluated using the area under the curve (AUC). To determine the threshold

Table 1 AUC values and minimum training presence threshold values for the LIG, LGM and present models for the three studied species

Species	Time	AUC (±SD)	Min train pres (±SD)
<i>C. polyrhiza</i>	LIG	0.83 ± 0.04	0.08 ± 0.02
	LGM	0.81 ± 0.04	0.11 ± 0.03
	Present	0.82 ± 0.04	0.10 ± 0.03
<i>C. cineraria</i>	LIG	0.82 ± 0.04	0.14 ± 0.02
	LGM	0.83 ± 0.04	0.09 ± 0.02
	Present	0.83 ± 0.04	0.07 ± 0.05
<i>C. caeruleus</i>	LIG	0.81 ± 0.05	0.19 ± 0.03
	LGM	0.81 ± 0.05	0.20 ± 0.06
	Present	0.78 ± 0.06	0.20 ± 0.05

value for each prediction, we used the value of the minimum training presence logistic threshold (Table 1).

Floral phenotype

Using collections from 57 natural populations of *C. polyrhiza*, we calculated the population average of the critical floral fit-related trait (i.e. throat length), which is the distance between the elaiophore and the fertile parts (Fig. 1a). In each sampled population, one to three freshly open flowers were measured from 10 to 51 individuals. Our morphological dataset comprised populations from Cosacov et al. (2014), as well as additional populations, and covered the entire range of the species.

Relationship between potential stable areas of interaction and floral phenotypic variation

To identify geographic areas that presented high suitabilities and were shared between species through time, we first equalized the spatial resolution of the layers. Then, to obtain co-occurrence maps (i.e. areas of interaction) for each time period, we multiplied the layer of *C. polyrhiza* and that of each pollinator using the raster calculator in QGIS. To quantify areas of interaction in km² at each time point, on the interaction maps we counted the number of pixels with a probability of occurrence higher than 0 and converted them to km². Finally, to identify areas where the presence of the interactions was predicted for the three climatic scenarios, the LIG, LGM and present layers were multiplied using ENVI v4.8 (Exelis Visual Information Solutions, Boulder, CO, USA). Therefore, through this arithmetical operation, we obtained two maps with potential stable areas of each interaction over time.

For each set of interacting species, we generated a categorical map in ENVI using nonparametric decision tree analysis. This map was a visual representation of the relationship between spatial variation in throat length and persistence of the interaction over time. The decision tree classifier performs multistage classifications by using a series of binary decisions to place pixels into classes and can be used with ordinal as well as categorical attributes (Friedl and Brodley 1997; Elnaggar and Noller 2010). For this analysis, we used the map of stable areas of interaction for each bee species and a “throat length” raster, generated with the inverse distance weighting (IDW) spatial interpolation method of the 57 data points with morphological information. Based on previous studies (Cosacov 2010; Cosacov et al. 2014), throat length was classified as short (<10.76 mm) or long (≥10.76 mm), corresponding to the ecotypes previously reported. Classification rules were as follows: if throat length is short and the probability of the interaction with *C. caeruleus* > 0 and with *C. cineraria* = 0,

then we reclassified those cells as A; if throat length is short and the probability of the interaction with *C. caeruleus* > 0 and with *C. cineraria* > 0, we reclassified those cells as B; if throat length is long and probability of the interaction with *C. cineraria* > 0 and with *C. caeruleus* = 0, then we reclassified those cells as C; if throat length is long and probability of the interaction with *C. cineraria* > 0 and with *C. caeruleus* > 0, then we reclassified those cells as D.

Finally, to evaluate whether geographical variation of the measured floral trait was associated with a long-lasting interaction of *C. polyrhiza* with a specific species of bee, we performed a multiple regression. In this analysis, we used the probability of interaction through time for each bee as predictor and throat length as the response variable. The predictive power of this model on floral phenotype variation was tested with restricted maximum likelihood estimation (REML; Zuur et al. 2009), comparing the multiple regression with a null regression model. The analyses were performed with the *lme4* package (Bates and Maechler 2010) in R v.2.15.0.

Results

Species distribution modelling

The current SDMs for the studied species were a quite good representation of the extant species geographical distribution (Roig-Alsina 1999, 2000; Ehrhart 2000; Sérsic 2004; Cosacov et al. 2014).

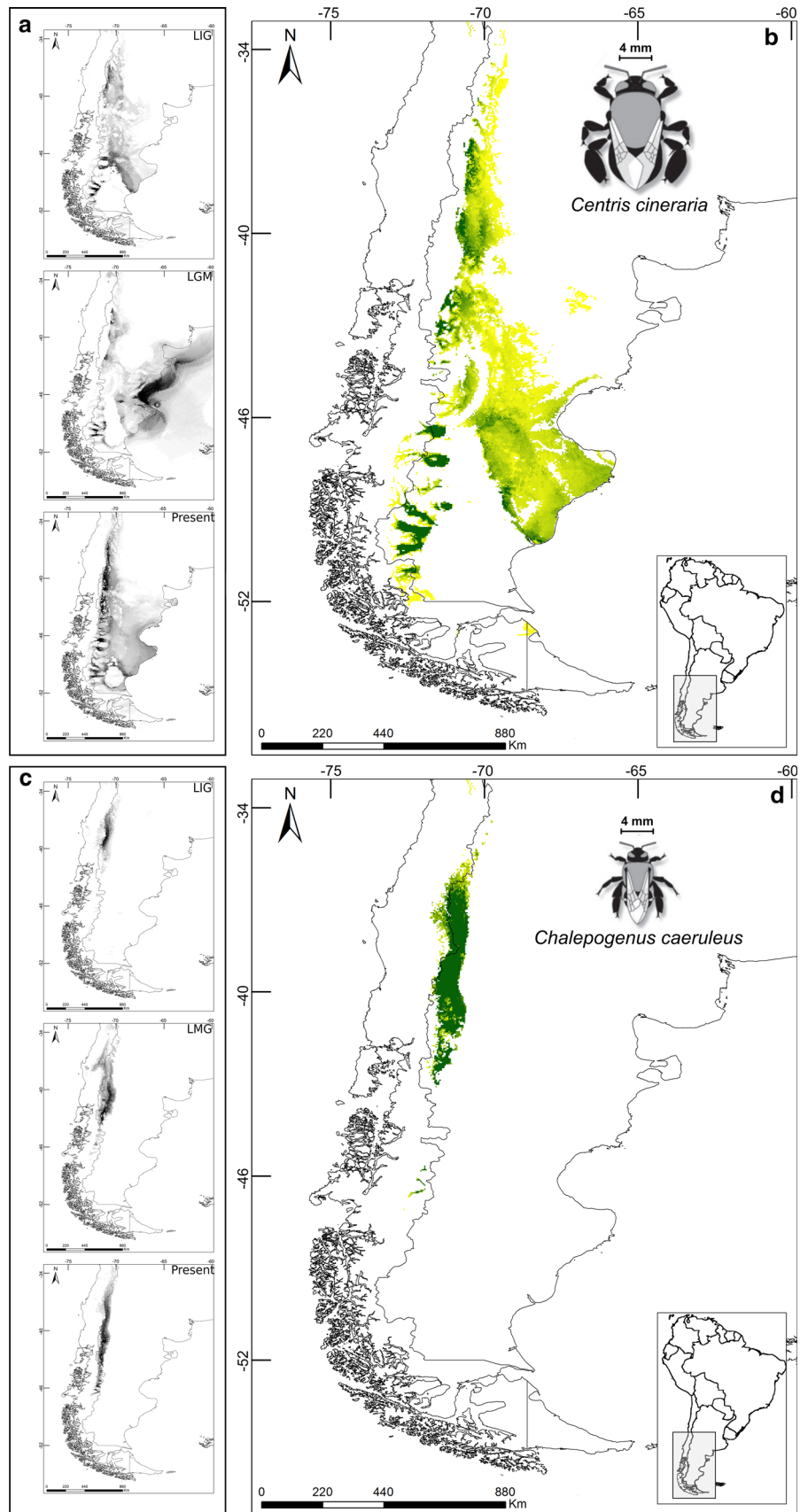
In addition, the mean values of AUC based on 10 replicates for all simulations were in average 0.82 (Table 1). The SDMs for each species at each time period are provided (Online Resource 2).

Potential stable areas of interaction

C. polyrhiza–*C. cineraria*

Under current conditions, we identified a continuous overlapping range along Patagonia. Here, the most suitable areas of interaction were present in the west (~71°W). The hindcasted area of interaction showed an important longitudinal shift across time (Fig. 2a). Under the LIG scenario, three centres of high climatic suitability for the interaction were recovered at around 71°W at three different latitudes: Neuquén (~40°–36°S), north-western (~46°S), and south-western Santa Cruz (~51°S). The northern and southernmost of these areas also had high suitabilities in the LGM hindcasted range, although their areas were smaller. Additionally, in the LGM hindcasted range, two new high climatic suitability areas were found: a small western (~42°S) and a coastal Patagonian zone (around San Jorge Gulf) (Fig. 2a).

Fig. 2 Modelled spatiotemporal dynamic of the interaction of *C. polyrhiza* and their pollinators, across Quaternary climatic fluctuations and the present climatic scenarios. **a** Stable areas of interaction of *C. polyrhiza* and *C. cineraria* for each time period. **b** Potential stable areas for the *C. polyrhiza*–*C. cineraria* interaction over time. **c** Stable areas of interaction of *C. polyrhiza* and *C. caeruleus* for each time period. **d** Potential stable areas of *C. polyrhiza*–*C. caeruleus* interaction over time. In all maps, dark hues indicate high probability of occurrence of the corresponding interaction



Stable areas (i.e. areas suitable through time; Fig. 2b) were found along the Andean slopes in a transitional zone between the steppe and the temperate Patagonian forest. In spite of an evident fragmented shared range during the LGM, the spatial extent of the total suitable area for the interaction is larger during the LGM than during the LIG and current scenarios (Fig. 3a).

C. polyrhiza–*C. caeruleus*

The inferred area of interaction was more stable for this interaction than for the one with *C. cineraria* (Figs. 2, 3a). This area showed a progressive latitudinal southward extension from the LIG to the present (Fig. 2c; from $\sim 38^{\circ}$ – 40° S to $\sim 38^{\circ}$ – 46° S). Stable areas extended between $\sim 35^{\circ}$ and 45° S (Fig. 2d) and were fairly continuous, only fragmented at the southernmost latitudes ($\sim 46^{\circ}$ S).

Relationship between potential stable areas of interaction and floral phenotypic variation

When we compared the probability of each area of interaction across time in relation to *C. polyrhiza* ecotypes, the interaction with *C. cineraria* was always more probable than the interaction with *C. caeruleus* in populations of *C. polyrhiza* with long throats, and was statistically significant for each time period (Fig. 3b). In contrast, the interaction of *C. caeruleus* with flowers in populations with short throats was always higher than that of *C. cineraria*, although it was only significantly different during the LGM (Fig. 3c).

Our nonparametric decision tree analysis (Fig. 4a) detected two main areas. The first one corresponds to regions where the morphotype with long throats predominates (areas of high probabilities of interaction between *C. polyrhiza* and *C. cineraria*). The second is the region presenting an association between the short throat morphotype and areas of high probabilities of interaction with *C. caeruleus*. A thin stripe between these two areas showed an admixture of morphotypes. Here, there is high probability of interaction with both bees, independent of the length of the throat.

The multiple regression analysis was significant ($F = 7.65$, $R^2 = 0.22$, $p = 0.0012$) and indicated a significant correlation between floral phenotype and the stability of interacting areas in the last 120,000 years. In addition, this model differed significantly of the null regression model when compared through REML ($P = 0.0012$). The multiple regression model ($y = 11.43 + 298.43x_1 - 72.88x_2$) was used to provide a map that shows the phenotype prediction, which is highly congruent with the decision tree map (Fig. 4b).

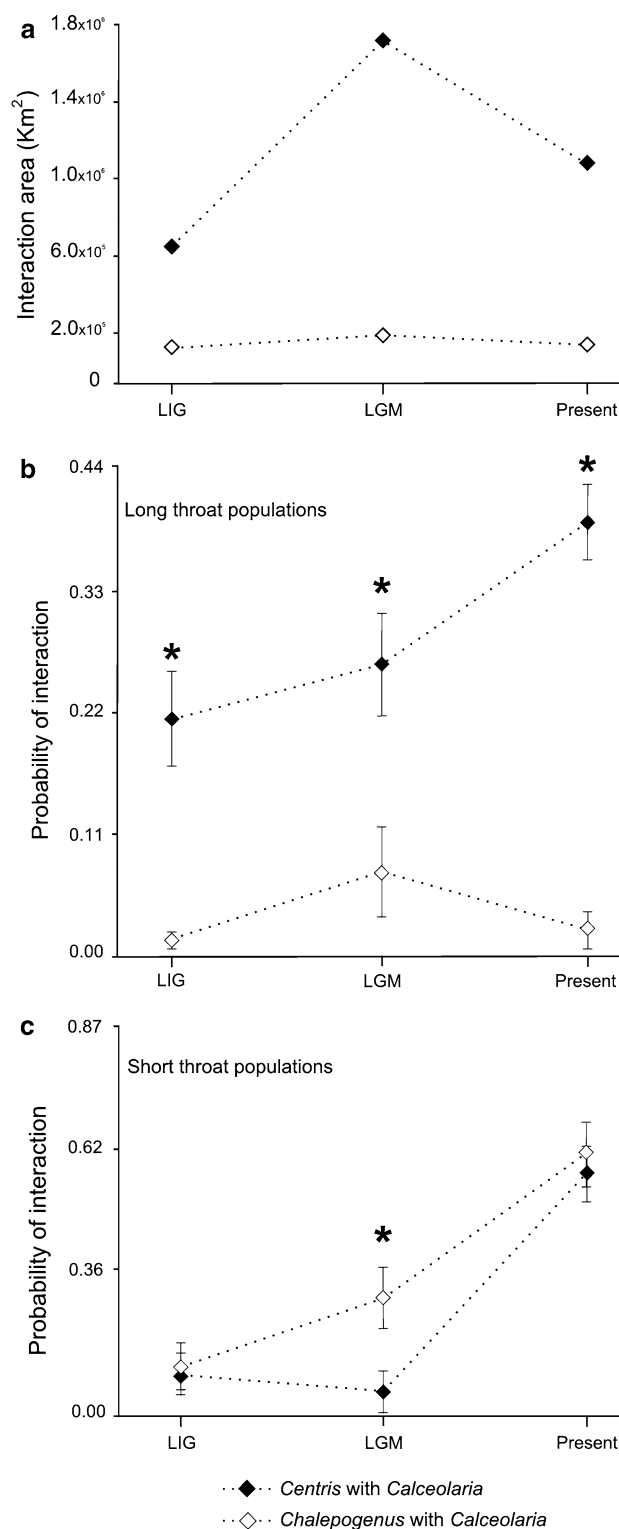


Fig. 3 a Changes in the area of interaction between *C. polyrhiza* and their pollinators (km²) from the Last Interglacial (LIG) to the present. b Changes in the probability of interaction of each *C. polyrhiza* pollinator with the long throat populations from the LIG to the present. c Changes in the probability of interaction of each *C. polyrhiza* pollinator with the short throat floral ecotype populations from the LIG to the present. * $p < 0.05$ between pollinators for each period

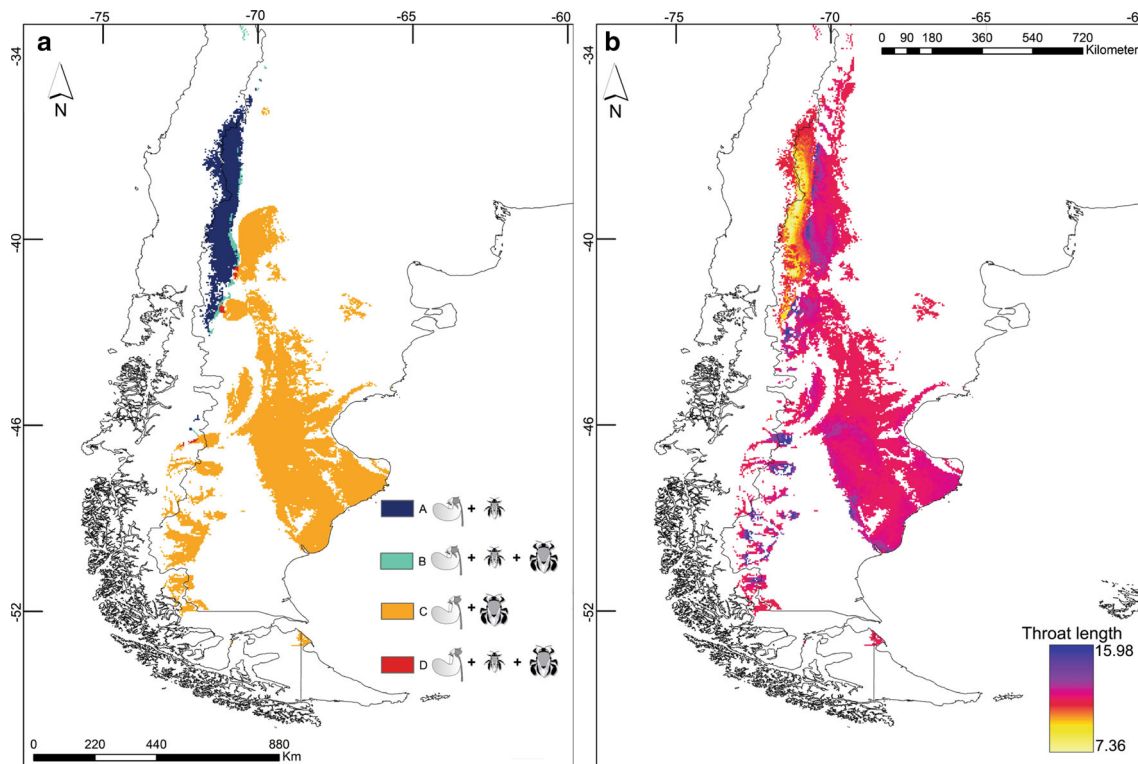


Fig. 4 a Multiple regression model ($y = 11.43 + 298.43x - 72.88y$) projected into a map showing the phenotypic prediction based on the inferred interaction of *Calceolaria polyrhiza* with its pollinators in the last 120,000 years. A = short throat + *C. caeruleus* > 0 + of *C. cineraria* = 0. B = short throat + *C. caeruleus* > 0 + *C. cineraria* > 0.

C = long throat + *C. cineraria* > 0 + *C. caeruleus* = 0. *D* = long throat + *C. cineraria* > 0 + *C. caeruleus* > 0. **b** Nonparametric decision tree analysis showing the relationship between throat length spatial variation and interactions persistence for each pollinator over time

Discussion

Many studies of geographical variation of phenotypic traits have provided insights into the role of pollinators as major drivers of evolutionary differentiation (e.g. Benitez-Vieyra et al. 2006; Baranzelli et al. 2014a; Peter and Johnson 2014). Although there is a consensus that Quaternary environmental changes substantially impacted the landscape and promoted rapid evolutionary changes in many species (Hewitt 2000; Tribsch and Schonswetter 2003; Hosner et al. 2014), analysis of phenotypic variation in plants has usually neglected the underlying historical context (but see Rodríguez-Gómez et al. 2013; Baranzelli et al. 2014b; Maubecin et al. 2016). In this study, we associated species palaeodistribution models and phenotypic variation by analysing the floral phenotype divergence of *C. polyrhiza* in relation to stable areas, where the species would have persisted with different pollinators during the last 120,000 years.

Overall, our models showed two distinct areas where the interaction with one of the bee species predominated. This was shown for *C. cineraria* across the Patagonian steppe and for *C. caeruleus* in the Patagonian forest. Although the presence records of both bee species show evident

superposition in the Andean forest ecoregion, predicted areas for both interactions overlapped only in some localities, situated in a restricted longitudinal zone at around 71°W and between 38° and 40°S. This is in agreement with our field observations: although *C. cineraria* is usually observed in forest regions, in those areas, 92% of the total floral visits are done by *C. caeruleus* (Cosacov et al. 2014). Our results thus reinforce the idea that there is some differentiation in the climatic preference between these two bee species. Along these lines, a previous study indicated that localities where plants are visited by *C. caeruleus* or *C. cineraria* do differ in their climatic and edaphic features (Cosacov 2010). These niche preferences in the two genera have been also observed at a continental scale in subtropical and temperate zones. Indeed, while *Centris* are mainly found in plains and dry habitats, *Chalepogenus* occur in montane and more humid areas (Roig-Alsina 1999, 2000; Cocucci et al. 2000; Cosacov et al. 2008).

Our SDMs suggest that past climatic changes affected the interaction between *C. polyrhiza* and both bee species in time and space, although these effects differed among bee species. On the one hand, the interaction with *C. caeruleus* appears to have been spatially stable and maintained in the Andean-Patagonian forest. Although the

interaction shows latitudinal shifts and southward expansions from the LIG to the present, we found no evidence of strong spatial fragmentation or range contractions. On the other hand, the interaction of *C. polyrhiza* with *C. cineraria* appeared to have been strongly affected by past climatic changes. This was shown by remarkable changes in the extent of the predicted area, which was the largest during the LGM. As expected, this interaction predominated in the Patagonian steppe. However, during the LIG the interaction persisted mainly in fragmented areas between 46° and 52°S and probably also between 35° and 40°S. During the LGM, the areas of interaction experienced a strong contraction, coupled with an eastward shift towards the exposed Patagonian shelf of the Atlantic coast. The area of interaction appears to have shifted again in current projections, this time towards the Andes. Taken together, these results suggest that the interaction with *C. cineraria* would have been affected by important spatial shifts through time.

It is interesting to point out that many phylogeographic and palaeobotanical studies support the idea that in the past these two vegetation types communities were present before the last 120,000 years analysed in our study (Wilf et al. 2005; Okuda et al. 2006; Sérsic et al. 2011). In particular, several studies support the existence of Patagonian steppe since ~4 Ma (Barreda et al. 2008; Jakob et al. 2009; Sede et al. 2012; Cosacov et al. 2013; Nicola et al. 2014) and the existence of Andean-Patagonian forest at least since Eocene (Leppe et al. 2012; Acosta et al. 2014).

Although we did identify strong spatial shifts, we could also detect some regions that remained stable across the three time periods considered. The localization of the most probable persistent interacting areas is highly coincident with the four glacial refugia proposed for *C. polyrhiza* based on a previous phylogeographic study (Cosacov et al. 2010). In this framework, the putative plant refugia located in south-western Santa Cruz (51°S), central-western Chubut (43°S), and the steppe San Jorge Gulf area would correspond to regions where *C. polyrhiza* would have survived interacting with *C. cineraria*. The refugium located at 40°S coincided with a zone predicted as stable for both interactions. Cosacov et al. (2010) also proposed small, isolated, valley refugia northern than 40°S, and along the high Andean grasslands, where our projections predict a spatiotemporally stable interaction with *C. caeruleus*. Importantly, the inferred historically stable areas, as well as the eastward expansion and subsequent retraction of interacting areas with *C. cineraria*, are patterns highly congruent with phylogeographic patterns revealed in *C. polyrhiza* and in many other Patagonian terrestrial organisms (reviewed in Sérsic et al. 2011), thus further supporting our palaeoclimatic reconstructions.

Although the interaction with *C. cineraria* was observed in almost all current stepparian populations of *C. polyrhiza*,

our results suggest that historical events significantly altered the range of these two mutualistic partners. For *C. caeruleus*, the inferred scenario was drastically different since the interacting area with this species remained practically unaltered across time. Detection of stable areas is relevant to the study of phenotypic evolution because plant populations that persisted in situ with their pollinators during past climatic change could have remained closer to adaptive peaks, compared with places where mutualists started interacting only in recent periods. Stable areas would thus be appropriate to explore the possible link between floral phenotype divergence and a long-lasting interaction with different pollinators.

Our results suggest that the phenotypic divergence observed in *C. polyrhiza* could be the result of two different pollination regimes. Using two different statistical approaches (a decision tree and a multiple regression analysis), we show a clear association between long and short throat populations of *C. polyrhiza* and a historical stable interaction with *C. cineraria* and *C. caeruleus*, respectively. These two bee species greatly differ in body size, which is an important trait involved in effective pollination because it fits the throat length of the flower. Because *C. cineraria* is at least twice as large as *C. caeruleus*, it is an efficient pollinator of both floral ecotypes, while *C. caeruleus* can only pollinate the short one (Cosacov 2010; Cosacov et al. 2014), a prediction confirmed by previous field observations (Cosacov et al. 2014). Despite the fact that our models predicted an association between *C. caeruleus* with long throat populations in the transitional area between the steppe and Andean forest, these populations corresponded *on average* to the long throat morphotype, and although both morphotypes coexist in these localities (Cosacov 2010), *Chalepogenus* was never observed there. Related to this inconsistency, it should be noted that our analyses could have missed factors other than climate (see above), which could have also influenced species ranges (Wisiz et al. 2013; Anderson 2016).

We also observed that the oldest lineages of *C. polyrhiza* are located in areas where a high probability of stable interaction with *C. caeruleus* was inferred from the LIG. Further, these lineages consist of populations with short throat morphotypes. Comparing the present results with those from our previous phylogeographic study (Cosacov et al., 2010), it is worth noting that the areas of stable interaction with *C. caeruleus* corresponded to localities occupied by two different plant lineages. On the one hand, the high Andean populations of *C. polyrhiza* were recovered as an old genetic group predating 1 My, while more recent lineages of *C. polyrhiza* were also recovered, showing a westward shift into the Andean forests as a result of a postglacial colonization from the Patagonian

steppe. Interestingly, these observations show an independent phenotype convergence within the evolutionary history of *C. polyrhiza*.

Our study is one of the few to explore the dynamics of two specialized mutualists across space and time and in relation to differentiation of the plant floral phenotype. Our results suggest that the response of *C. polyrhiza* to past climatic variation would have been modulated by the interaction with its mutualists, leaving a footprint on its floral morphology. Future studies using mutualists in comparative phylogeographic studies will allow us to further understand the evolutionary history of these interactions.

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