Research Article

Morphological and distributional patterns of native and invasive Trifolium (Papilionoideae, Leguminosae) species in southern South America

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The ability of a species to colonize new geographical areas is closely related to its morphological response to environmental gradients. Previous studies compared native and alien invasive species in their ability to modify their morphology in reaction to climatic and edaphic factors; however, taxonomically close species have been scarcely analyzed. In this study, morphological variability patterns associated with abiotic factors were evaluated in Trifolium polymorphum, a native species distributed from southern Brazil to northern Patagonia, and Trifolium repens, a species introduced as an important forage crop and naturalized in diverse environments of southern South America. A morphometric study was carried out using herbarium specimens that were then georeferenced to obtain environmental and distributional data. Morphological traits along abiotic and geographical gradients were analyzed, predictive distribution and current and future niches were modelled. Our results showed that leaf morphology of T. repens responds to precipitation and temperature factors, which could explain its invasive behavior in a wide range of environments. Morphological variability of the native T. polymorphum presented a low association with abiotic factors, suggesting possible difficulties in adapting to future environmental changes. Ecological niche modelling analysis showed several overlap areas between species in the current model and a greater expansion of the distribution range of the invasive species in future climate change scenarios.

Key words: abiotic drivers, climate change, invasiveness, legume, morphometry, niche modelling

Introduction

Invasive species usually show high phenotypic plasticity (Pigliucci et al., 2006; Sultan, 2001), in general of greater magnitude than their native congeners (Dickson et al., 2012). In heterogeneous environments, this plasticity would be mediated by the capacity for morphological response, generating intraspecific trait variation (Albert et al., 2011). Describing this response capacity is crucial to predict changes in the distribution of species and the floristic composition of plant communities in a global climate change context (Lande, 2009). Plasticity mechanisms would explain the success of a small percentage of introduced species that behave as invasive until they become naturalized (Richardson & Pysek, 2006). Strong associations between abiotic factors and morphological trait variability reveal the existence of a crucial mechanism, phenotypic plasticity, for generating environmental adaptability (Sattarian et al.,...
which could explain how alien species maintain fitness in stressful situations and/or increase fitness in favorable environments (Richards et al., 2006).

A global mean temperature rise of more than 2°C is predicted by the end of the 21st century (IPCC 2014). Plant species are sessile organisms; therefore, they are more susceptible to environmental changes than other organisms (Matesanz et al., 2010) and their specific distribution controls their evolutionary process and conservation status (Crisci et al., 1991; Elith & Leathwick, 2009). In southern South America, climate change predictions for the end of the century indicate increases in precipitation and mean annual temperature (Chou et al., 2014; Moreira de Melo et al., 2015) along with longer periods of droughts and floods (Kapsomenakis et al., 2011).

These climatic changes will modify productivity of plants (Reddy et al., 2010), affecting their distribution range (Lenoir & Svenning, 2015) and the species composition of ecosystems (Thuiller et al., 2008a; Van der Putten et al., 2010). Rapid climate change could be an advantageous context for invasive plants, since it is assumed that they have a greater capacity to adapt to a wide range of environments and that they will be able to withstand fluctuations (Bradley et al., 2010; Dickson et al., 2012; Liu et al., 2017; Matesanz et al., 2010). Moreover, invasive species will increase their dominance as climate change stresses their neighbors (Dukes & Mooney, 1999), impacting the ecosystem by generating competition for resources and thereby threatening native species that occupy similar niches (Catford et al., 2018). Invasive species are likely to respond in ways that can be predictable and some of these responses will be distinct from those of native congeners (Thuiller et al., 2008a). However, many studies have shown that, under a climate change scenario, some invasive alien species inhibit their expansion (Bellard et al., 2018; Merow et al., 2017; Sorte et al., 2013).

Legume species inhabit areas at different latitudes and with very diverse climates and soils (Lewis et al., 2005) and show significant phenotypic plasticity (Etterson, 2004; Piairo et al., 2012; Pichancourt & van Klinken, 2012; Pohlman et al., 2005; Robbiati et al., 2017; Zhmud & Dorogina, 2015). Southern South America is home to at least 12 native Trifolium L. species, and more than 20 have been introduced (Brako & Zarucchi, 1993; Zuloaga et al., 2019).

Trifolium species are annual, biennial, or perennial herbs, frequently symbiotic with Rhizobium bacteria. Leaves are alternate, trifoliate and have membranous stipules (Vincent & Butterworth, 2017). White, yellow, pink or purplish flowers are gathered in capituliform inflorescences, and some species can present cleistogamous flowers. The fruit is an utricle that encloses 1-4 seeds. T. polymorphum is considered a forage resource with low productivity, being tolerant to high use intensities due to a hypogeous seed bank (Burkart, 1987; Izaguirre, 1995) generated by its cleistogamous flowers (Speroni et al., 2010). It grows on landscape relief summit, stony or sandy-humiferous soils, tolerating temporary waterlogging (Izaguirre, 1995) from March to December. It is distributed in Uruguay, southern Brazil, southern Paraguay, center-eastern, northeastern to northern Patagonia, in Argentina, and northern to central Chile. On the other hand, T. repens, native to Europe, North Africa, and western Asia, was introduced to South America as forage for animal consumption due to its high protein content during the winter-spring growing season in temperate and temperate-cold climates (Sturite et al., 2007). It has a perennial cycle and is highly adapted to grazing due to its propagation by stolons and natural replanting (Ganderast, 2001).

The methodological approach of evaluating exotic and native species using large regional data sets has been used in many investigations (Hamilton et al., 2005; Muth & Pigliucci, 2006; Sutherland, 2004) and the trait approach for studying ecological insights is also commonly employed (Albert et al., 2011; Helsen et al., 2017). The integration of knowledge of phenotypic plasticity pattern and ecological niche models allow a better understanding of distributional changes resulting from climate change (Nicotra et al., 2010; Robbiati et al., 2017). Phenotypic plasticity could influence plant species distribution given it is related to the ability of the species to adapt to new conditions and buffer population declines (Thuiller et al., 2008b). In parallel, the knowledge of plastic traits is necessary to understand and predict plant responses to invasive species and global warming (Lavorel & Garnier, 2002) since it is known that they could increase the probability of population survival in the face of climate change (Henn et al., 2018; Nicotra et al., 2010).

To contribute to the understanding of a possible relationship between morphological and ecological response to abiotic factors and the capacity to invade new environments, we selected two Trifolium species: a) T. polymorphum Poir., endemic, and b) T. repens L., introduced as animal fodder. Trifolium repens is known to exhibit phenotypic plasticity related to environmental factors in other continents of the world (Caradus et al., 2006; Fazlioglu et al., 2018; Frankow-Lindberg, 2001; Huber et al., 2008; Weijischede et al., 2006; 2008) and locally in Chile (Acuña et al., 2019; Inostroza et al., 2018). Trifolium polymorphum is one of the most widely distributed of the group of native species in...
southern South America and shares with *T. repens* growth season and environments. Thus they occupy a similar ecological niche, with their occurrence overlapping in large areas of their distribution range (Burkart, 1952; Zohary & Heller, 1984). Thus, these species are excellent models to investigate and compare morphological variation associated with abiotic factors in a native and an invasive species in southern South America. Studying congeneric species can reveal traits associated with invasiveness and the possible generation of differentiated niches to avoid competition (MacDougall et al., 2009).

Based on these antecedents, we postulated two hypotheses. Firstly, we hypothesized that the invasive species *Trifolium repens* would present a higher morphological response in one or more morphological traits to one or more environmental factors than *T. polymorphum*, a native species with a comparatively limited distribution. This would explain part of its success as an invasive species; Secondly, we hypothesized that it is feasible to observe that the invasive species generates a displacement or overlapping of the ecological niche of the native species, responding more favorably to climate change. The aims of this work were (1) to explore the relationships between morphological responses to abiotic factors and invasiveness of a naturalized species versus its native congener; (2) to investigate if abiotic or geographical factors can reveal patterns of morphological variation in these species; (3) to identify plastic traits likely to be determinant in plant response to abiotic factors; and (4) to reveal if ecological niches of both species overlap and to predict distribution changes in a future climate change scenario. To meet these objectives we conducted data analyses to reveal the associations of morphological variation and abiotic factors. We also used ecological niche modelling (ENM) to understand the influence of environmental conditions on geographical distribution and to detect the possible overlaps of ecological niches in both present and future climate change scenarios.

### Material and methods

#### Study area

The study area extends from 21-55°S and 50-73°W (approximately 4,000,000 km²) in southern South America, and includes disparate climates, soils, altitudes and ecological communities. This contrast is fully manifested in the existing phytogeographic provinces (Cabrera, 1976; Oyarzabal et al., 2018). According to Cabrera (1994), in this area there are two phytogeographic regions, the Neotropical and Antarctic, which contain four phytogeographic domains, the Amazonian, Chaqueno and Patagonica-Andean; and the Subantarctic. A region that presents such drastic, contrasting and wide abiotic gradients, ranging from humid hot jungles, subtropical savanna, grasslands and scrublands to cold forest and tundra in the south of South America, allows for investigation of almost all the possible patterns of adaptive morphological variability of species.

#### Plant species and morphological characters

Because many traits vary significantly over the course of plant development and growth, environment-induced variation can be studied by measuring them at a common point of ontogeny (Evans, 1972). Given this, morphometry was performed on fully expanded leaflets and fully developed flowers. We measure 245 individuals from herbarium specimens (200 of *T. repens* and 45 of *T. polymorphum*) in BAA, BAB, CTES, LP and SI (herbarium abbreviations from http://sweetgum.nybg.org/ih/; Supplemental Appendix S1). In order to identify relevant characters in the adaptation of the species to the environment, we measured six vegetative and three reproductive characters (Table 1). Measurements were made using a binocular microscope OLYMPUS SZX9 with a light source V-LUX 1000.

#### Climatic, edaphic and geographical distribution data

Coordinates were obtained from herbarium labels; when this information was not available, the herbarium specimens were geo-referenced using Google Earth 8.0 (http://www.google.com/earth/index.html) according to Zermoglio et al. (2020) georeferencing protocol. Association analysis between morphological variability and climatic variables was carried out using monthly averages of climatic factors as they are considered to have a great biological impact and because monthly data was available: maximum temperature (TMAX) [°C], minimum temperature (TMIN) [°C], radiation (RAD) [kJ/m²×dia⁻¹] and precipitation (PP) [mm].

| Character Abbreviation Unit |
|-----------------------------|
| **Character** | **Abbreviation** | **Unit** |
| Leaflet length | LL | mm |
| Leaflet width | LW | mm |
| Internodes length | IL | mm |
| Petiole length | PL | mm |
| Stipule length | SL | mm |
| Peduncle length | PDL | mm |
| Flowers per inflorescence | FI | number of flowers |
| Flower length | FL | mm |

### Table 1. List of morphometric characters used in the study.
extracted from the WorldClim database (Hijmans et al., 2005) at a resolution of 30 arc second, and potential evapotranspiration (PET) [mm] extracted from the Global Potential Evapotranspiration database (Zomer et al., 2008). We generated two additional climatic variables: thermal amplitude (TA) [°C] and aridity index (AI), where $TA = TMAX − TMIN$ and $IA = PP/PET$. All climatic factors were analysed seasonally and annually. In addition, we included soil data using five edaphic variables: bulk density (BLDFIE) [kg m$^{-3}$], clay content (CLYPPT) [weight %], coarse fragment volumetric (CRFVOL) [volumetric %], soil organic carbon content (ORCDRC) [g × kg$^{-1}$], soil pH (PHIHOX), proportion of silt particles ($>0.002 \text{mm and} \leq 0.05 \text{mm}$) in the fine earth fraction (SLTPPT) [g/100g (%)] and the proportion of sand particles ($>0.05 \text{mm}$) in the fine earth fraction (SNDDPT) [g/100g (%)] extracted from SOILGRIDS$^{\text{TM}}$ (https://www.isric.org) at 2.5 arc minute spatial resolution.

For niche modelling, we used elevation and 19 bioclimatic variables extracted from WorldClim 2 database (http://www.worldclim.org) at a resolution of 2.5 arc minute (Fick & Hijmans, 2017) and soil data previously selected. To minimize biased fitting of the models produced by covariance among variables and consequent misleading results, we reduced some data because of multicolinearity. We performed a Pearson’s Correlation to identify pairs of variables with a high degree of correlation ($r > 0.8$). We selected the following climatic variables: Bio1 (Annual Mean Temperature), Bio2 (Mean Diurnal Range), Bio3 (Isothermality), Bio6 (Minimum Temperature of Coldest Month), Bio7 (Temperature Annual Range), Bio9 (Mean Temperature of Driest Quarter), Bio12 (Annual Precipitation), Bio15 (Precipitation Seasonality) plus elevation. Correlation tests were performed using INFOSTAT 2.0 (Di Rienzo et al., 2018).

To construct future projections, we used the RCP 6.0 (Representative Concentration Pathways) scenario for year 2070 (2060–2080) proposed by The Intergovernmental Panel on Climate Change (IPCC 2014), in which emissions peak around 2080, then decline, while CO2 levels rise to 670 ppm by 2100 and for South America a temperature increase of 1.5-2.5°C is projected (Torres et al., 2022). Given that each Atmosphere Ocean Global Circulation Models (AOGCMs) introduce uncertainty (Diniz-Filho et al., 2009; Nori et al., 2011), we selected three different models: CCSM4 (Community Climate System Model, version 4), CGCM3 (Fourth Generation Atmospheric General Circulation Model) and HadGEM2 (Hadley Centre Global Environmental Model, version 2) and used the average of selected bioclimatic variables of these three AOGCMs.

**Statistical analysis of morphological and abiotic data association**

A morphometric characterization was performed for both species. Means, standard deviations, coefficients of variation (CVs), quartiles and outliers were represented in boxplots and in Supplemental Table S1. We performed linear regression to explore possible relationships between environmental variables and morphological traits. In addition, we performed Mantel tests using INFOSTAT (Di Rienzo et al., 2018) separately correlating the matrix of vegetative morphology (MORPHVEG) and reproductive morphology (MORPHREP) with the matrix of geographic distances between individuals (GEODIST) and with each matrix of abiotic factors. Dissimilarity matrices of morphological traits were calculated using Euclidean distances among each pair of individuals. Geographical distances between each pair of individuals were calculated using QGIS 3.12 (QGIS.org, QGIS Development Team, 2021).

To detect geographical patterns of vegetative morphological variation in *T. repens*, we carried out a Principal Component Analysis (PCA). In order to clarify possible patterns, we mapped two putative morphotypes. Discriminant analysis and Kruskal-Wallis test were conducted to examine trait contribution and statistically significant differences in morphology between the two morphotypes. We are aware that some traits and abiotic factors in this study are by definition intercorrelated, and therefore the results must be interpreted individually. Even so, we used this approach because each of them may have slightly different ecological significance.

**Ecological niche modelling**

Ecological niche modelling (ENM) was employed to evaluate the present and future potential distribution of the two *Trifolium* species. Two hundred fifty-eight occurrences (45 of *T. polymorphum* and 213 of *T. repens*) were compiled using coordinates obtained for climatic analyses (Supplemental Appendix S1). ENM models were run in MaxEnt using the following settings: convergence threshold = 10 – 5, maximum iterations = 20 000, regularization multiplier = 1, replicates = 10, and replicates run type = crossvalidation. MaxEnt uses only species presence data and performs well with small sample sizes (Hernandez et al., 2006; Phillips et al., 2006). Models were then evaluated by the area under the receiver-operating characteristic curve (AUC)
(Lobo et al., 2008; Peterson, 2007). AUC scores were first calculated using all records and then using 75% training vs. 25% testing data sets (Fielding & Bell, 1997). A jackknife test was also run to evaluate the relative importance of the variables.

The projections were stored in ASCII raster format and imported to QGIS to plot the maps using the average values of the thresholds provided by MaxEnt. These thresholds are represented by different colors to indicate the predicted probability that environmental conditions were suitable, with values of 1–0.8 (dark red) for the highest predicted probability, 0.8–0.6 (red) for high predicted probability, 0.6–0.4 (blush) for medium predicted probability, 0.4–0.2 (light blush) for a low predicted probability and 0.2–0.0 (white) for very low predicted probability. Finally, the MaxEnt projections were reclassified to convert the continuous output into a presence/absence (0 indicating niche dissimilarity, 1 indicating high similarity between niches, and values close to 0.5; p: <0.001). The TA factor did not show high or significant levels of association with morphological traits variation. Regarding the hydric factors, seasonal spring and summer precipitations were the most relevant for T. repens morphology, being the leaflet traits the ones that most responded to the pluviometric gradient (Fig. 3 and Supplemental Table S2). When taking annual precipitation, the low levels of association of winter and autumn precipitation reduce the coefficients from $R^2 < 0.4$ to $R^2 < 0.1$, in the case of leaflet traits. Mantel test results using complete seasonal and annual environmental factors are shown in Supplemental Tables S3 and S4 and a selection of most relevant results is presented in Table 2 (using vegetative matrix) and in Table 3 (using reproductive matrix). No association was found for T. polymorphum morphological variation, both vegetative and reproductive, with environmental abiotic factors. On the other hand, associations were found between the vegetative morphological variation of T. repens and several climatic factors. The TMINAnnual and the PPSpring and PPSummer were the most relevant and significant factors. For the two species no association was found between morphology and edaphic factors.

Analysing 200 T. repens specimens, two putative morphotypes were found. PCA shows two overlapping groups (Fig. 4) based on the morphometry of vegetative traits. On a map (Fig. 1) the geographic pattern of these

**Morphological response to abiotic factors**

Linear regression analyses revealed that no abiotic factor, neither climatic or edaphic, was significant as an explanatory factor for T. polymorphum morphometric variability (Fig. 3 and Supplemental Table S2). By contrast, in the case of T. repens, some significant positive associations were found: mainly with thermal and precipitation factors. TMIN was the most relevant factor to explain T. repens morphometric variability, especially for the foliar traits LL and LW ($R^2 > 0.5$; $p: <0.001$), being also seasonally relevant ($R^2 > 0.5$; $p: <0.001$). TMIN also showed a relevant level of positive association to IL and lower with the rest of the vegetative traits, being almost null for the reproductive traits. In the case of TMAX, the level of association was lower, both annually and seasonally for most of the traits, although it remained relevant for foliar traits ($R^2 > 0.35$; $p: <0.001$). The TA factor did not show high or significant levels of association with morphological traits variation. Regarding the hydric factors, seasonal spring and summer precipitations were the most relevant for T. repens morphology, being the leaflet traits the ones that most responded to the pluviometric gradient (Fig. 3 and Supplemental Table S2). When taking annual precipitation, the low levels of association of winter and autumn precipitation reduce the coefficients from $R^2 < 0.4$ to $R^2 < 0.1$, in the case of leaflet traits. Mantel test results using complete seasonal and annual environmental factors are shown in Supplemental Tables S3 and S4 and a selection of most relevant results is presented in Table 2 (using vegetative matrix) and in Table 3 (using reproductive matrix). No association was found for T. polymorphum morphological variation, both vegetative and reproductive, with environmental abiotic factors. On the other hand, associations were found between the vegetative morphological variation of T. repens and several climatic factors. The TMINAnnual and the PPSpring and PPSummer were the most relevant and significant factors. For the two species no association was found between morphology and edaphic factors.

**Results**

**Distributional and morphometric characterization**

The difference in distributional range between the native and the invasive species was evidenced after mapping the studied specimens (Fig. 1). *Trifolium repens* is widely distributed in Southern South America, being found in the tundra and forest of Patagonia, in the drylands of Argentina, Chile and Bolivia, in grasslands and savannahs, and associated with subtropical jungle vegetation. In contrast, *T. polymorphum* distribution is concentrated in the humid subtropical grasslands and shrublands of central-eastern Argentina, western Uruguay and southern Paraguay. Morphometric characterization for both species showed both higher phenotypic values and variation levels in *T. repens* than in the native *Trifolium* species (Fig. 2, Supplemental Table S1). Most variable traits for both species were IL, PL, PH (>50 CV) while SL, FI, FL showed low levels of variability (<30 CV). Leaflet traits (LL and LW) and PDL showed intermediate values of variability (30-50 CV). Great differences in terms of CV occurred between species in PH, LL, LW, PDL, SL and FI, always being higher in *T. repens*. The highest differences were registered in the PH, SL, PL and FL traits, while the values did not differ greatly in FI, IL and LW, LL and PDL.
morphotypes was evidenced. The dividing line in the geographic distribution of *T. repens* showed morphotype 1, represented in Fig. 1 west of the dividing line and with red dots in Fig. 4; and morphotype 2, represented in Fig. 1 to the east and with blue dots in Fig. 4. Discriminant analysis showed that LL, IL, and LW were the traits that contributed to differentiate the two groups, while the rest of the traits did not contribute to the discrimination of the morphotypes (Supplemental Table S5). A high proportion of observations were correctly placed in each group (96.63% for morphotype 1 and 95.37% for morphotype 2). Kruskal-Wallis test showed that there was a statistically significant difference between the two putative morphotypes in all vegetative traits and IL, but not for FL and FI, evidencing higher morphometric values for all traits in morphotype 2 (Supplemental Table S6).

**Ecological niche modelling**

Current and future potential distributions of *T. polymorphum* and *T. repens* based on the Maxent algorithm are presented in Fig. 5. ENM results indicate that the models performed well (using all records and training-testing data with AUC > 0.95). The AUC for each group was better than random (i.e., model AUC values exceeded the 95th percentile of the null AUC distributions).

The potential distribution range for *T. polymorphum* in current climatic conditions comprises a total of 639,954 km², and the distribution projection model was
a fairly good representation of the extant geographic distribution. The variables isothermality (Bio3), annual precipitation (Bio12) and bulk density (BLDFIE) made the greatest contributions (Supplemental Table S7). The optimal values of these variables were as follows: isothermality ranged from 31.15 to 95.38, annual precipitation ranged from 0 to 2000 mm and bulk density ranged from 285 to 1560 (kg m\(^{-3}\)). For future prediction reach a total of 1,079,380 km\(^2\), expanding its distribution mainly in the northern area.

The potential distribution range of *Trifolium repens* in current conditions comprises 1,818,640 km\(^2\) and the distribution projection model was a fairly good representation of the extant geographical distribution (although slightly over-predicts, resulting in a continuous geographical distribution). The variables isothermality (Bio3) and minimum temperature of the coldest month (Bio6) made the greatest contributions (Supplemental Table S8). The optimal values of these variables were as follows: isothermality ranged from 30.73 to 97.87 and minimum temperature of coldest month ranged from 17.6 to 23.41. The future prediction reached a total of 2,625,500 km\(^2\), expanding its distribution mainly in the southern area, although an

Fig. 2. Box plots representing the mean, median, interquartile range, adjacent values (lines), and outliers (dots) of quantitative characters in *T. repens* and *T. polymorphum*.
expansion is observed to the north, even exceeding the study area, up to 18ºC14
S. Climatic niche overlaps results indicate medium to close relationships in terms of climatic similarity between T. polymorphum and T. repens (D: 0.47, I: 0.74).

Discussion

Morphological response to environmental factors

Numerous works have studied morphological responses of different plant species to abiotic environmental factors (Carroll et al., 2001; Chalcoff et al., 2008; Helsen et al., 2017; Nattero et al., 2011; Paiaro et al., 2012). However, few studies have focused on species with a close phylogenetic relationship and with important morphological similarities (Burns, 2004; Cuda et al.,

Table 2. Selected Mantel tests results of correlation among T. polymorphum and T. repens vegetative morphometric matrix of dissimilarities, geographical distance, climatic and soil factors.

| Matrix          | T. polymorphum | T. repens |
|-----------------|----------------|-----------|
| A               | B              | R         | P         | R         | P         |
| MORPHVEG GEODIST| −0.06          | 0.716     | 0.19      | 0.001     |
| MORPHVEG TMINAnnual | 0.00           | 0.999     | 0.28      | 0.001     |
| MORPHVEG TMAXAnnual | 0.00           | 0.999     | 0.14      | 0.001     |
| MORPHVEG PPAnnual | 0.00           | 0.999     | 0.01      | 0.533     |
| MORPHVEG PPSpring | 0.00           | 0.495     | 0.20      | 0.001     |
| MORPHVEG PPSummer | 0.02           | 0.298     | 0.21      | 0.000     |
| MORPHVEG RADAnnual | −0.01          | 0.368     | 0.07      | 0.064     |
| MORPHVEG PETAnnual | −0.05          | 0.722     | 0.06      | 0.060     |
| MORPHVEG TAannual | −0.03          | 0.535     | −0.01     | 0.734     |
| MORPHVEG AIAnnual | −0.01          | 0.399     | −0.04     | 0.776     |
| MORPHVEG BLDFIE | −0.03          | 0.801     | 0.02      | 0.406     |
| MORPHVEG CLYPPT | −0.04          | 0.724     | 0.03      | 0.197     |
| MORPHVEG CRFVOL | 0.06           | 0.115     | 0.08      | 0.000     |
| MORPHVEG ORCDRC | −0.09          | 0.840     | 0.02      | 0.326     |
| MORPHVEG PHIHOX | 0.04           | 0.246     | 0.01      | 0.297     |
| MORPHVEG SLTPPT | −0.10          | 0.931     | 0.09      | 0.066     |
| MORPHVEG SNDPPT | −0.09          | 0.919     | 0.07      | 0.016     |

Note: A and B are the two matrices tested, R is the Mantel statistic which expresses the level of correlation between matrices and P shows the p-value indicating statistical significance with an alpha of 0.05.
Furthermore, most previous works recreate natural variability and environmental gradients in greenhouse trials, which may fail to comprehensively analyze the effects of environmental heterogeneity. Our approach using herbarium specimens and previously collected environmental data, is not as common (Paiaro et al., 2012; Pérez-Barrales et al., 2009; Scrivanti et al., 2014; Van Kleunen et al., 2010).

Plant species able to change leaf size when humidity or temperature becomes a limiting factor are able to survive in restrictive and changing environments (Crescente & Gratani, 2013; Gratani & Crescente, 1997; Pohlman et al., 2005; Souto et al., 2009; Wright et al., 2005). Traits associated with resource capture are known to be more variable than others, such as reproductive traits (Violle et al., 2009). If there are clinal variation patterns of morphological traits along environmental gradients, a strong association between these variables defined by the regression coefficient is to be expected (Barton, 1999). We found evidence for morphological variation in *Trifolium repens* vegetative traits in response to climatic factors, since this species shows a positive relationship between leaflet size variation and both temperature and precipitation (Fig. 2). Leaf traits should therefore be considered plastic traits, and temperature and Spring and Summer precipitation should be considered environmental drivers of its morphology. On the other hand, the lack of association between morphological traits and abiotic factors in *T. polymorphum* could constrain the ability of populations to react to future environmental changes (Gratani et al., 2012). This lack of association could be due to the loss of phenotypic plasticity resulting from a process of specialization to adapt to specific environments (Fazlioglu et al., 2018). It is also possible that non-invasive species, which exhibit less phenotypic plasticity, have a certain homeostatic capacity to adapt to changes in abiotic factors (Davidson et al., 2011). Our results agree with previous studies showing that temperature has a stronger and more positive effect on invasive species’ vegetative morphology than on that of native species (Liu et al., 2016). While the native species has a long evolutionary history in its thermal environment, the invasive species could be pre-adapted to a variable and wide-range thermal environment (Bradley et al., 2015).

Regarding the results of seasonal precipitation, the differential influence of spring-summer versus winter-
Fig. 5. Current and predictive distribution models for *T. polymorphum* (1) and *T. repens* (2).
autumn precipitation in *Trifolium repens* morphology could be related to the annual growth cycle and inactivity of this perennial species. A positive relationship between spring-summer precipitation and leaflet values could be explained by limited leaflet growth in spring and a hydric stress during the summer.

Petiole and internode length have been described as plastic traits in *Trifolium repens* that respond to shade, photoperiod, cold temperature stress and interspecific competition (Acuña et al., 2019; Fazlioglu et al., 2018; Frankow-Lindberg, 1999; Huber et al., 2008; Wejschedé et al., 2006; 2008). However, we did not find strong associations between these traits and radiation or other abiotic factors. A low level of association was observed between these traits and minimum temperature and precipitation which suggests a response of plant architecture to the environment (Guo et al., 2007).

Soil nutrients, specifically phosphorus, have been identified as determinant factors for *T. repens* internode and leaf morphology (Caradus et al., 2006); therefore, this topic should be included in a subsequent study. In fact, none of the edaphic factors incorporated in our work showed an association with morphological variability in either species, suggesting that the distribution of species is limited by climatic conditions rather than by edaphic factors.

We found morphological variability in reproductive characters, especially inflorescence peduncle length (Fig. 2), and a low degree of association with abiotic factors in both species (Supplemental Table S2). Vegetative reproduction could be compensating for a deficit in reproductive capacity due to the lack of plasticity in reproductive morphological traits. However, variability in reproductive morphology could be explained by the influence of not only the studied abiotic factors (Lambrecht & Dawson, 2007), but also by biotic ones which were not included in our study, such as pollinating fauna (Helsen et al., 2017; Lambrecht & Dawson, 2007; Pélabon et al., 2011; Strauss & Whittall, 2006).

Since *T. repens* is a self-incompatible species and obligatorily pollinated by insects, especially bees (Rodet et al., 1998), our results corroborate Berg’s hypothesis that floral morphology is decoupled from vegetative variability, constrained by the need to maintain a stable and proportionate relationship with pollinators (Berg, 1959 & 1960). This confirms that plasticity or morphological response to the environment must be studied individually for each trait (Pélabon et al., 2011; Wagner et al., 2007). In the case of *T. polymorphum*, the underground flowers are cleistogamous, necessarily self-pollinated, and the aerial flowers are chasmogamous, allogamous or self-pollinated (Speroni et al., 2009). However, according to Real et al. (2007), it should be considered cross-pollinated by bees, so the same criterion can be applied to *T. repens*.

It has been shown that morphotypes growing in diverse habitats along a climatic gradient do not have a pattern of ecologically related genetic differentiation, and that morphological and physiological differences are due to phenotypic plasticity (Nahum et al., 2008). Introduced species are frequently less genetically variable in the new environment due to the genetic bottleneck and drift experienced by the small founding populations (Barrett & Richardson, 1986). In the case of *T. repens*, no significant levels of genetic variability have been found around the world (Olsen et al., 2007) and this has been corroborated in southern Chile (Acuña et al., 2019; Inostroza et al., 2018). In addition, there are records from introduction zones where the morphophysiological diversity of the populations was not related to molecular diversity, but was explained by evolutionary adaptation to the environments of origin (Annicchiarico & Carelli, 2014). Since *T. repens* was introduced in the different environments of the study area, we suggest that the phenotypic plasticity of the species has allowed it to adapt to local conditions by generating morphotypes.

For both species, Mantel tests showed a lower level of association between morphological traits and abiotic factors than linear regressions. This could be due to the fact that in the regressions a single trait was analysed and in the Mantel tests the vegetative or reproductive matrix was used, which gave less sensitivity to specific associations between morphology and the studied environmental factors. We must also highlight the importance of biotic and anthropogenic factors that have not been included in our work and that could have an important role in the morphological variability of these species (Helsen et al., 2017; Morris et al., 2020). In any case, to interpret our findings, it is important to consider that each of the abiotic factors studied are components of a single environment in which both climatic and soil factors interact, and that the final outcome is rarely the result of additive partial effects (Dieleman et al., 2012; Juntila et al., 1990; Liu et al., 2016).

**Distributional patterns and climate change impact**

ENM analysis revealed that isothermality (Bio3) has the greatest influence on distribution in both *Trifolium* species. However, both have a wide isothermal range. The distribution of *T. polymorphum* is further limited by annual precipitation and bulk density, whereas *T. repens* distribution is further constrained by minimum temperature of coldest month. *Trifolium polymorphum* has been
described as a species that preferentially grows in high, rocky, sandy soils, and does not tolerate floods (Burkart, 1987). Moreover, since a fraction of its seed production occurs underground, soils with high density could be another limiting factor. Previous works have shown that minimum temperatures and cold stress reduce the phenotypic values of T. repens and increase plant mortality (Collins et al., 1997; Frankow-Lindberg, 1999). Our results suggest that beyond limiting growth, extreme temperatures in the coldest month restrict the ability of this species to respond plastically and limits its distribution. Genes and vegetative traits associated with tolerance to abiotic stress and low temperatures have been identified in populations in the Patagonia region (Acuña et al., 2019; Inostroza et al., 2018). However, no molecular study has addressed distributions in contrasting environments such as that here. Regarding the hydric factors, under a scenario of increased precipitations in the distributional range of both species, and considering that invasive species are typically favored in environments with greater water availability (Cavaleri & Sack, 2010; Liu et al., 2016; Sorte et al., 2013), T. repens can be expected to take advantage of its morphological response capacity, whereas T. polymorphum cannot.

ENM analysis showed expansion of the potential distribution range in future projections in both species, but proportionally greater in T. repens, which could increase the risk of competition between these species. Although these species seem to have similar climatic requirements and area overlap, the presence of T. polymorphum would be consolidated north of its distribution range, a region where T. repens would be less frequent. However, T. repens is used as forage for animal consumption and its introduction to new areas by human action is possible. Rapid climate change can act as a factor of high impact on the natural selection of species, favoring those that are more plastic phenotypically (Dukes, 2007) and contributing to the displacement of native species (Funk, 2008). A high level of phenotypic response to environmental factors suggests the ability to invade a broad ecological range (Hiatt & Flory, 2020), increasing the chances of a speciation event (Lütge, 2006; Nonaka et al., 2015; Rieseberg & Willis, 2007). Those plant species that can expand their ecological niche (Chevin et al., 2010; Sultan, 2001) may be able to overcome the effects of global warming (de Villemereuil et al., 2018).

In conclusion, we demonstrated differences in the morphometric response capacity to abiotic environmental variability of the invasive species T. repens as compared to the native species T. polymorphum. This capacity was evidenced in several vegetative traits in response to thermal and seasonal precipitation gradients across the extensive environmental heterogeneity of southern South America. These results can partially explain the ecological behaviour and dispersion of both species. Morphological variability of T. polymorphum, although at similar levels to its congener, was not associated with any climatic or edaphic factors studied. As is known, the lack of capacity to respond to climatic variables suggests difficulties in adapting to climate change. Nevertheless our projection of the potential distributional range of T. polymorphum shows expansion, as it does for T. repens, suggesting that environmental changes may not seriously affect the native species, while continuing to favour the invasive species.

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