Intraspecific phylogeny of a Patagonian fescue: differentiation at molecular markers and morphological traits suggests hybridization at peripheral populations

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- **Background and Aims** Grasses of the Festuca genus have complex phylogenetic relations due to morphological similarities among species and inter-specific hybridization processes. Within Patagonian fescues, information concerning phylogenetic relationships is very scarce. In *Festuca pallescens*, a widely distributed species, the high phenotypic variability and the occurrence of interspecific hybridization preclude a clear identification of the populations. Given the relevance of natural rangelands for livestock production and their high degradation due to climate change, conservation actions are needed and knowledge about genetic variation required.

- **Methods** To ravel out the intraspecific phylogenetic relations and to detect genetic differences, we studied 21 populations of the species along its natural geographic distribution by coupling both molecular (ITS and *trn*L-F markers) and morpho-anatomical analyses. Bayesian inference, maximum likelihood, and maximum parsimony methods were applied to assemble a phylogenetic tree, including other native species. The morphological data set was analysed by discriminant and cluster analyses.

- **Key Results** The combined information of the Bayesian tree (ITS marker), the geographic distribution of haplotype variants (*trn*L-F marker) and the morpho-anatomical traits, distinguished populations located at the margins of the distribution. Some of the variants detected were shared with other sympatric species of fescues.

- **Conclusions** These results suggest the occurrence of hybridization processes between species of the genus at peripheral sites characterized by suboptimal conditions, which might be key to the survival of these populations.

Key words: intraspecific genetic differentiation, phylogenetic relationships, ITS, chloroplast DNA markers, morpho-anatomical analyses, plant traits, *Festuca pallescens*, *Festuca*, Patagonia, glacial refugia, hybridization, speciation.

INTRODUCTION

Dryland ecosystems cover almost half of Earth's land surface and house nearly 40% of humanity, with an outstanding importance for socio economic activities (Bradford et al., 2020; Maestre, 2021). Natural rangelands that develop in dryland areas constitute most of the world's grasslands and are degraded due to overexploitation (Grau and Aide, 2008; James et al., 2013). In addition, plant communities of dryland ecosystems are highly sensitive to changes in temperature and precipitation regimes, such as those occurring due to global climate change (Magrin et al., 2014; Golodets et al., 2015). Increasing the knowledge of evolutionary processes between populations of key grassland species is necessary to both help predict upcoming changes and contribute to solutions.

Broadly distributed grassland species, covering environmental gradients and heterogeneous habitats, provide an interesting experimental design to study evolution and adaptation to suboptimal conditions (Maestre et al., 2012). Within these habitats, species have adapted in unique ways by developing deep and efficient root systems, water store structures, or by going dormant during dry periods, to evade, avoid or resist drought (Davies et al., 2012; Moreno and Bertiller, 2015). These species tend to present large phenotypic and genetic variation (Manel et al., 2012). The level of genetic differentiation among central populations of the same species tends to be low due to historical persistence and the constant genetic exchange between individuals, but habitat heterogeneity could result in genetically distinct ecotypes (Hufford and Mazer, 2003; Eckert et al., 2008; Jakob et al., 2009). The continuous stress that populations undergo in dryland ecosystems may lead to adaptive changes and eventually even speciation processes (Hoffmann and Hercus, 2000). Natural hybridization may be a mechanism to promote adaptation, particularly in small and isolated populations that develop near the limits of their distribution range (Thompson et al., 2010). Specifically, glacial refugia during the Quaternary could have developed the conditions for the occurrence of ancient hybridization processes (Liu et al., 2018; Shepherd et al., 2022). Ultimately, hybridization may lead to adaptive evolution and speciation which allows the new species to colonize extreme habitats to which neither of the parental species are adapted (Rieseberg et al., 2003; An-tian et al., 2011). The recognition of ecotypes or hybrids can be complex, but the use of molecular markers complemented by the analysis of morphological character expression permits the reconstruction of phylogenetic trees (Pourebrahimi et al., 2022), thus allowing to assign taxonomic status.

The Patagonian region constitutes the largest arid or semiarid ecosystem in southern South America. Climatic gradients mainly determined by the Andean mountains and a complex geomorphology give rise to a variety of environments, soil and vegetation types (Villalba et al., 2003; Gaitán et al., 2020). Plant communities in Patagonia are represented by two main functional groups: shrubs and perennial grasses (Bertiller and Bisigato, 1998). Natural rangelands dominated by tussock grasses and scattered shrubs are the main input for cattle raising and have been intensively used since the early 1900's (Defossé and Bertiller, 1990). Due to overgrazing and the effects of climate change, these valuable habitats undergo degradation and desertification, meaning that key species of grasses become less abundant due to a drastic reduction of their populations (Gaitán et al., 2017; Oliva et al., 2020). As perennial dominant grasses lose habitat, grasslands become deteriorated and different species, particularly shrubs, may establish and dominate (Gonzalez and Ghermandi, 2021).

Festuca pallescens (St.-Yves) Parodi is a dominant allohexaploid species from natural rangelands of Patagonia (Dubcovsky and Martínez, 1992). This keystone species has a wide distribution and inhabits many diverse environments. It has been well studied and described morphologically and physiologically (Somlo et al., 1985; Bertiller et al., 1990; Fernández et al., 2004, 2006; Caballé et al., 2011; López et al., 2019, 2021), but information about its genetic variability is scarce (Dubcovsky and Martínez, 1991, 1992; López et al., 2020).

Phenotypic variation among populations analysed in a common garden was observed as well as the molecular identification of a hybrid ecotype (López et al., 2018, 2020). This ecotype found at a peripheral population clustered with another closely related polyploid, *Festuca argentina* (Speg.) Parodi, a sympatric species of *F. pallescens* (López et al., 2018). However, this previous study covered only part of the distribution range. Here, by extending the analysis to the whole natural range, we aim to analyse intraspecific variation and to reconstruct interspecific phylogenies involving other native species. Our main hypothesis is that the wide distribution range of this species, covering highly heterogeneous habitats, promoted genetic differentiation among populations. We also hypothesise that hybridization with sympatric species is possible on peripheral areas. We combined a molecular study of populations encompassing the whole distribution range, with a thorough morphological analysis to describe intraspecific variation.

MATERIALS AND METHODS

Sampling strategy

Twenty-one populations of *F. pallescens* covering the distribution range of the species were analyzed (Table 1, Fig. 1). Leaves were collected for DNA extraction and sequencing. Sampled individuals were identified in the field based on exomorphological characters described for the species (Nicora, 1978; Catalán and Müller, 2012). Additionally, four individuals of the sympatric hexaploid species *Festuca gracillima* Hook. F., from the southernmost area of *F. pallescens'* distribution, were collected and included in the analysis.

DNA extraction and PCR amplification

DNA from at least three individuals per population was extracted. Leaf tissue was frozen using liquid nitrogen and grounded to fine powder with an automatic mixer mill (Resch, Germany). DNA extraction followed the protocol of Doyle and Doyle (1987), with slight modifications (Gonzalo-Turpin and Hazard, 2009). Two DNA markers were used for the phylogenetic analysis: the internal transcribed spacer (ITS) and a chloroplast DNA region (*trn*L-F). The complete ITS region (ITS1-5.8S-ITS2) was amplified using the primers CY1-CY3 (Wright et al., 2006), approximately 600 to 700 bp of length (Torrecilla and Catalán, 2002; Catalán et al., 2004). For the amplification we used 40 ng of DNA as template, 0.625 U of GoTaq DNA polymerase (Promega, Madison, WI, USA) with 1X Colorless GoTaq® reaction buffer (Promega), 1.5 mM of $MgCl_2$, 0.25 mM of each dNTP, and 0.3 µM of each primer in a total volume of 30 µL. PCR reactions were carried out following this program: 4:30 min at 95 °C, 30 cycles of 30 s at 94 °C, 1 min at 56 °C and 2 min at 72 °C, and a final extension of 10 min at 72 °C. The *trn*L-F region (~1000 bp, Catalán et al., 2004) was amplified using the universal primers c and f (Taberlet et al., 1991) with 100 ng of DNA, 1U of GoTaq DNA polymerase (Promega, Madison, WI, USA) with 1X Colorless GoTaq® reaction buffer (Promega), 2 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.2 µM of each primer in a total volume of 50 µL. The amplification program used for this marker was as follows: 1 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C, followed by a final extension of 7 min at 72 °C. PCR products for both markers were checked for positive amplification in 1.5% agarose gels, stained with Gel Red, and visualized with a UV transillumінator. Amplified regions yielded bands of approximately 700 bp (ITS) and 1000 bp (*trn*L-F) which were purified using the ExoSAP-IT^{TM} PCR Product Cleanup (Thermo Fisher) commercial kit and then sequenced in a capillary sequencer (ABI 3700, Unidad de Genómica, Instituto de Biotecnología de INTA, Hurlingham, Argentina).

Phylogenetic Analyses

The ITS sequences were aligned using Muscle with manual adjustments when needed in AliView 1.26 (Larsson, 2014). For the phylogenetic analysis of the ITS region we additionally used sequences from 14 Patagonian species of *Festuca* retrieved from Genbank (Supplementary data Table S1: species and GenBank accessions). In addition, a sequence from the ecotype of *F. argentina* previously mentioned was also included (López et al., 2018). Two Patagonian grasses (*Poa ligularis* Nees ex Steud. var. ligularis, and *Pappostipa speciosa* var. speciosa Trin. et Rupr.) were used as outgroups. All the ITS and *trn*L-F sequences *of F. pallescens* and *F. gracillima* were deposited in Genbank (Accession numbers are provided in Supplementary data Table S1).

A matrix of 24 sequences of 578 characters each was conformed for the analysis of the ITS region. Gaps were treated as missing data, and indels as point mutations. Boundaries of each sequence were established by the alignment with the species of *Festuca* retrieved from Genbank (14 species). To assess the phylogenetic relationships between species of Patagonia and among populations of *F. pallescens* analyses based on Bayesian Inference (BI), Maximum Parsimony (MP) and Maximum Likelihood (ML) criterion were run. The IB, MP and ML analyses were carried out with MrBayes 3.2.7a (Ronquist et al., 2012), TNT 1.5 (Goloboff and Catalano, 2016) and the online software RAxML (Kozlov et al., 2019), respectively. We used JModelTest 2.1.10 to establish the best fitted nucleotide substitution model that is required to run BI and MV analyses (Darriba et al., 2012). This resulted in model GTR+G according to both the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC). Bayesian analyses were carried out by running pre-stablished parameters (1 million generations initiated from different random trees, sampling every 100th generations model parameters such as nucleotide substitution rates, gamma shape, proportion of invariable sites, nucleotide frequency) estimated by MrBayes. After ‘burn-in’ (discarding 25% of the total sampling), parameters were sampled when reaching stationary. The ITS consensus tree, obtained from 15,000 trees, was supported by values branches from the posterior probability (PP). Clades with more than 95% PP values are considered well supported. Parsimony analyses were executed by subjecting the data set to a heuristic search strategy to find all equally parsimonious trees (MULPARSon, TBR) with 10,000 replicates, saving no more than 10 trees of length equal or shorter than 10 (López et al., 2018). These saved trees were collapsed to a consensus tree that was used as a negative constraint for a second search. All parsimonious trees obtained were used to compute a strict and 50% majority rule consensus tree. Bootstrap support for branches was calculated through heuristic searches of 1,000 replicates. Clades with bootstrap values under 50 are shown unresolved. We used the software FigTree (http://tree.bio.ed.ac.uk/software/figtree/) to edit and visualize the consensus tree. To reconstruct the relationships between haplotypes we run the program Network 10.2.0.0 which works with a median-joining network algorithm (Bandelt et al., 1999). The dataset used for the reconstruction contained the chloroplast DNA sequences of *F. pallescens* (seven haplotypes), *F. gracillima*, *F. purpurascens* Banks and Sol. ex Hook. f., *F. argentina* and the sequence corresponding to the hybrid ecotype (López et al., 2018).

Morphological data

Twelve populations of *F. pallescens* that differ on either of the two evaluated molecular markers were selected for morphological characterization (Table 1). Plant material was obtained from the living collection of *F. pallescens* populations held at the Campo Experimental Agroforestal Trevelin (CEAT INTA Esquel; 43°07´30´´S, 71°33´04´´W, 356 masl), Argentina. Twenty-four quantitative and nine qualitative morphological traits were measured in six to twelve specimens of each population (Supplementary data Table S2).

Morphological characters included in this study are those traditionally used for the delimitation of species in *Festuca* (Dubcovsky and Martínez, 1988; Catalán and Müller, 2012). In this genus, the distribution of sclerenchyma in the leaf cross section is a main trait for species identification (Aiken et al., 1985; Dubcovsky and Martínez, 1988). Therefore, leaf anatomy characters were observed in the middle region of the blade of the penultimate leaf of an innovation in all specimens studied. The identification of different combinations of adaxial and/or abaxial girders of sclerenchyma of vascular bundles was based on leaf cross section stained with safranin.

Statistical analysis

The quantitative variables were tested for normality with the Shapiro-Wilks test (Mahibbur and Govindarajulu, 1997), as well as using diagram boxes. Homogeneity of variance was checked with Bartlett's test. One-way ANOVA ($\alpha= 0.05$) was performed to evaluate the significance of the differences among the specimens for each trait. A Tukey's test ($p< 0.05$) was applied for *a posteriori* comparison of each pair of means. For the analysis of the variable lemma awn length, ACA population (1) was excluded because plants lack awns in the lemma (see results).

The morphological data set (Supplementary data Table S3) was analysed by discriminant and cluster analyses, with each character represented by mean values. Morphological character values were standardized prior to use in multivariate analyses. Pearson and Spearman correlation coefficients were estimated to identify pairs of highly correlated characters that may distort multivariate analyses (Michener and Sokal, 1957; Conover, 1999). Discriminant analysis allows classifying samples within predefined groups using the discriminant functions adjusted for maximizing the between-groups to within-groups ratio of variance. Cluster analysis was performed to reveal the structure residing in the morphological dataset. Sample similarities using the morphological data matrix were calculated based on the Euclidian distance and the average linkage hierarchical agglomerative method was used to establish clusters. The statistical analyses were performed using Infostat v. 2015 (Di Rienzo et al., 2015).

RESULTS

Variation at the ITS region

Six variants were identified at the ITS region along the distribution range of *F. pallescens*. The most common variant (F.pallescensVar1) was detected in 14 populations. Four northern populations (ACA (1), LMI (2), CHO (3) and HUI (4)) shared a distinctive ITS sequence, and the other four variants were found on two western (CRO (13) and FON (16)), one eastern (RMA (17)), and one southern population (GAI (21)) (Fig. 1a). The ITS dataset included 19 taxa: *F. pallescens* (with six variants), 16 native species of *Festuca*, including the hybrid ecotype of *F. argentina* (Supplementary data Table S1), and two outgroups (*P. ligularis* and *P. speciosa*). From the 578 aligned nucleotide positions, 73 (12.63%) resulted parsimony informative. The three phylogenetic analyses exhibited similar topologies, with two major clades that separate the *F. pallescens* variants from the hybrid ecotype. The BI tree showed the highest support values (Fig. 1a), MP and ML can be found at supplementary materials (Supplementary data Fig. S1). The ITS variants found at *F. pallescens* specimens conformed a well-supported clade, associated to *F. gracillima*. A strong association with other Patagonian native species (e.g., *F. purpurascens*) and with northern species (e.g., *F. ventanicola*) is also observed. In a separate clade, the hybrid ecotype variant clustered together with *F. argentina*. This clade is sister to only one species distributed at the extreme north of Argentina (*F. superba*).

Fig. 1

Variation at the chloroplast DNA (trnL-F) region

A total of seven chloroplast haplotypes were found among the analyzed populations. The topology of the network shows the ancestral character of the most frequent haplotype (Hp1) (Fig. 2). Haplotype 4 shares the same six nucleotide long insertion as *F. purpurascens* but differs by two nucleotides in other positions. Haplotypes 2 and 5 share an insertion of 22 nucleotides but differ on two positions. One of these polymorphisms is an ambiguity (W in IUPAC code) with Hp2 showing W, and the two alternative nucleotides observed in Hp1, Hp5, Hp6 and Hp7 (T), and Hp3 and Hp4 (A). Haplotype 7 shows high similarity with *F. gracillima*´s haplotype and distanced from Hp1 by nine mutations. Additionally, the sympatric species *F. argentina* differs from Hp1 by 13 mutated positions and shares the same haplotype as the hybrid ecotype (Fig. 2).

The most frequent haplotype (Hp1) was found in 18 populations (Fig. 2). Haplotypes were fixed in 17 populations, 15 exhibiting the common haplotype and the other two (BLE (20) and GAI (21)), exclusive types. Intra-population variation was observed in four populations (CLI (5), JAC (9), YAG (11) and RMA (17)) (Fig. 2).

Fig. 2

Morphological analyses

Significant differences between two or more populations for twenty of the twenty-four continuous variables were detected (ANOVA; $p<0.05$). The average values and standard deviation of the quantitative traits analyzed, as well as the results of a *posteriori* test, are summarised in Supplementary data Table S2. Population ACA (1), from northern Patagonia, was clearly the most differentiated. Its specimens showed ciliated ligules with significantly shorter hairs ($p < 0.05$). Northern populations (ACA (1), CLI (5), PIL (7), HUI (4) and JAC (9)) presented shorter hairs in the abaxial surface of leaves compared to southern populations (APE (12), CRO (13), FON (16), RMA (17), LBA (18), BLE (20) and GAI (21)). Specimens of populations ACA (1) and HUI (4) presented longer ligules and were different compared to other populations ($p < 0.05$). In addition, populations ACA (1), HUI (4), CLI (5) and JAC (9) showed significantly shorter spikelet ($p < 0.0001$). Two populations of south Patagonia (LBA (20) and GAI (21)) had longer synflorescences ($p < 0.0001$). Plants from CRO (13) and FON (16) populations presented longer lodicules ($p < 0.0001$).

Most variables were not correlated, except for spikelet length - lemma length (Pearson= 0.83), spikelet length - palea length (Pearson= 0.87), and lemma length - palea length (Pearson= 0.9). Pearson and Spearman correlation values were similar. Since the use of highly correlated characters is an implicit weighting of these characters and suggest potential multicollinearity problems, the total number of variables was reduced to 30 for final analyses (Supplementary data Table S3).

Discriminant Analysis led to the identification of morphological patterns that allowed a good discrimination among some populations. Populations ACA (1) and HUI (4) were clearly discriminated when the first two discriminant functions (axis 1 and axis 2) were plotted (Fig. 3), while ACA (1), HUI (4) and CLI (5) were discriminated considering the axes 1 and 3 (Supplementary data Fig. S2). The first three axes accounted for 62.82% of the variation. The absolute values of the coefficients of the standardised discriminant functions are shown in Supplementary data Table S3.

Fig. 3

Leaf cross section revealed the presence of sclerenchyma under the abaxial epidermis and in the ribs under the adaxial epidermis in all specimens analysed. In addition, different combinations of adaxial and/or abaxial girders of sclerenchyma in vascular bundles depending on the population were observed (Fig. 4). Population ACA (1) was the only one with double girders of sclerenchyma (adaxial to abaxial) in the main and secondary vascular bundles. In other populations the main and secondary vascular bundles showed different combinations with presence and/or absence of abaxial girders of sclerenchyma. In ACA (1) population all specimens showed abaxial girders of sclerenchyma in the main vascular bundle and 50% of them presented double girders. In secondary vascular bundles all plants also presented double girders of sclerenchyma. Populations that presented abaxial girders of sclerenchyma in the main vascular bundle were HUI (4), CLI (5), PIL (7), JAC (9), APE (12), CRO (13), RMA (17), and GAI (21) (see different percentages in Fig. 5). In populations FON (16), LBA (18) and BLE (20) girders of sclerenchyma in the main vascular bundle were not observed. Populations that presented abaxial girders of sclerenchyma in secondary vascular bundles were PIL (7), JAC (9), APE (12), CRO (13), and GAI (21) (see percentages in Fig. 5). In populations HUI (4), CLI (5), FON (16), RMA (17), LBA (18) and BLE (20), girders of sclerenchyma in secondary vascular bundles were not present.

Fig. 4

In the cluster analysis, populations were grouped in terms of their similarity according to the 30 morphological traits analysed (Supplementary data Table S3). Considering a reference line equal to 50% of the maximum distance (a frequently used criterion) to validate clusters, the cut-off line was established at distance 5.83. The dendrogram shows that ACA (1) population is clearly separated from the rest and populations of north and south Patagonia were grouped into two main clusters (Fig. 5).

Fig. 5

DISCUSSION

Maintaining genetic diversity across populations of a species is essential to ensure its survival, particularly under the current climate change context. We took the first steps towards describing differentiation among populations of a widespread keystone species of an arid and semiarid region. In this study, we found intraspecific variation at molecular markers and morpho-anatomical traits. Edge populations of *F. pallescens* hold different genetic variants in comparison to those located at the center of the distribution. Distinct populations can be observed particularly at northern, eastern and southern sites. In general, the same pattern is observed for both markers. Similarly, morphological traits also separated populations in two latitudinal groups.

Intraspecific phylogeny

The Poaceae family includes more than 700 genera of economic and ecological significance, but phylogenetic relationships among species are complex and sometimes unresolved (Grass Phylogeny Working Group, 2001; Soreng et al., 2017). In addition, phylogenetic studies encompassing the relationships among Patagonian fescues are limited

(Dubcovsky, 1988; Ospina, 2016; López et. al., 2018). The genus *Festuca* holds more than 500 species all over the world (Inda et al., 2008). Although well supported phylogenies exist, the representation of Patagonian species is very scarce, e.g., only *F. argentina*, *F. subantartica*, *F. purpurascens*, *F. gracillima*, *F. magellanica* and *F. pyrogea* were included (Torrecilla and Catalán, 2002; Torrecilla et al., 2003; Catalán et al., 2004; Inda et al., 2008; Minaya et al., 2017). In a previous study, we reported the position of *F. pallescens* within these phylogenies, clearly positioned within the "fine-leaved lineage" (López et al., 2018). Here, with an enlarged sampling, we reconstructed the relationships between the Patagonian fescue species and the *F. pallescens* variants. Our results show that most of *F. pallescens'* variants are closely related to *F. gracillima*, a sympatric species that occurs at the southernmost end of the distribution. This species is described as a complex together with *F. pallescens* (Dubcovsky and Martínez, 1991; Torrecilla and Catalán, 2002; Catalán and Müller, 2012). The variant detected in the four northernmost populations is separated from the main clade, conforming an unresolved polytomy to another sympatric species, *F. purpurascens*. The natural distribution of *F. purpurascens* overlaps with *F. pallescens'* distribution, and the two species showed high genetic similarities at both molecular markers. *Festuca gracillima*, *F. purpurascens* and all the variants of *F. pallescens* were grouped with most species of *Festuca*, and were separated, with a strong support, from a group that included *F. argentina*, *F. superba* and the hybrid ecotype. These results are consistent with Catalán et al. (2004), in which the festucoid grasses are separated in two mayor linages: "broad leaved" and "fine leaved" (Inda et al., 2014). *Festuca argentina* would belong to the first group, while *F. purpurascens* and *F. pallescens* would presumably belong to the latter (Catalán, 2004; Inda et al., 2008; López et al., 2018). In a further study of molecular, anatomic and micro-morphologic characters, these species are also separated in different groups (Ospina, 2016), but *F. argentina* is placed in an ambiguous position, close to either broad or fine leaved lineages. Even though Ospina (2016) placed *F. purpurascens* and *F. pallescens* in a fine leaved group according to his molecular results, anatomically he describes *F. purpurascens* within the broad leaved lineage while *F. pallescens* is placed in the fine leaved lineage together with *F. gracillima*. Clearly the high variation within and among species of the *Festuca* genus deserves more attention and further studies will contribute to a better comprehension of the relationships of the species belonging to this genus.

Genetic differentiation along the natural range

We identified eight distinct populations that differed from the most frequent ITS variant. Four of these populations are located at the northern edge of the distribution, and all the individuals sampled share the same sequence. This region was affected by sea introgressions and glaciations during the Miocene and Pleistocene (Ramos, 1982; Folguera and Ramos, 2002; Rabassa et al., 2005), which makes it a perfect spot for stochastic factors to develop differentiation from the central populations (Eckert et al., 2008). Within this northern region the extent of glaciations was limited to the foothills of the Andes (Flint and Fidalgo, 1964; Hollin and Schilling, 1981), leaving many ice free areas where species could survive in situ (e.g. Markgraf et al., 1995; Sérsic et al., 2011; Soliani et al., 2012). At this latitude, *F. pallescens* shares its habitat with other native species of the genus, which are morphologically similar (e.g., *F. acanthophylla* E. Desv. var. *acanthophylla* and *F. acanthophylla* var. *scabriuscula* (Phil.) J.C. Ospina, Aliscioni & S.S. Denham). Marginal populations are considered active regions for speciation processes which are particularly common in plants (Crawford et al., 2010; Rajakaruna, 2018). Ancient hybridization and speciation processes at glacial refugia might be a possible explanation to the variants shared by northern populations, but also for the southern variants found on this study (see next section).

Other four populations at the species range edges have shown distinct variants. The occurrence of differentiation at suboptimal locations could imply an evolutionary advantage under extreme climatic conditions, allowing the species to colonize and remain at these further frontiers. For instance, plant species increase their ploidy level with latitude (Rice et al., 2019) and most Patagonian fescues are mainly polyploids (Dubcovsky and Martínez, 1988, 1992). Polyploidy diversifies an organism's genetic background, provides selective advantages to novel or stressful environments (Van de Peer et al., 2017; Wei et al., 2019) and can also be a common way of sympatric speciation through hybridization processes (Wei et al., 2018; Gordon et al., 2020). Therefore, showing distinct genetic and morpho-anatomical variants, edge populations of *F. pallescens* could either suggest an ecological advantage (i.e., drought or salinity stress) or reinforce the possibility of hybridization with sympatric species.

A similar pattern of distribution of the genetic variation was displayed at the chloroplast level. The less frequent haplotypes found in our study, were detected in marginal populations (east and south of the distribution). The populations carrying the alternative variants match the distribution of different putative glacial refugia described for Patagonian plant species (Cosacov et al., 2010; Sérsic et al., 2011; Baranzelli et al., 2022) (Fig. 2). Two haplotypes found at the southernmost populations near one of the aforementioned refugia (Sérsic et al., 2011) are of particular interest, Hp4 and Hp7 from populations BLE (20) and GAI (21) respectively. The haplotype 4 shares a step mutation with one haplotype found in population RMA (17), closer to the center of the natural range for the species, but it also presents a six nucleotide long insertion that matches the sequence of *F. purpurascens*. On the other hand, Hp7 is almost an exact match of *F. gracillima's* sequence. The populations BLE (20) and GAI (21) belong to the southernmost region of the distribution, where it overlaps with the distribution range of *F. purpurascens* and *F. gracillima* (Dubkovsky and Martínez, 1987). Hybridization with other species of the genus that share the same ploidy count is plausible and it has been argued as either a cause or consequence of speciation (Dubkovsky and Martínez, 1991a, 1992; Oliva, 1996; Šmarda and Stančík, 2005; Ospina, 2016; Fernández et al., 2017). Fescues are known to hybridize within the genus (Humphreys et al., 1995; Inda et al., 2014), and a putative hybrid ecotype has already been described in a location with suboptimal conditions and isolated from the center of the distribution (López et al., 2018). Interspecific hybrids have also been described as common among other species of the Poaceae, and these events can complicate the reconstruction of phylogenetic relations between species (Díaz-Pérez et al., 2014; Baiakhmetov et al., 2020).

The high genetic diversity detected at RMA population (17) at both markers, deserves attention. This population is close to a described plant refuge and a phylogeographical break for several species reviewed by Sérsic et al. (2011). The steppe vegetation is poorly represented since most studies were devoted to temperate forests species. However, the in situ survival throughout glacial cycles was suggested based on phylogeography and ecological niche modelling that indicated stable geographic distribution of two *Hordeum* species for at least the Holocene (Jakob et al., 2009). Past vegetation reconstruction within the southern distribution range of *F. pallescens* confirms its presence during the Holocene (Echeverría et al., 2022). The legacy of the glacial era is still imprinted in the current taxa, but *F. pallescens* was never studied. Our results are the first information contributing to the knowledge of the evolutionary history of the species. Future studies should include other chloroplast markers to infer phylogeographical patterns.

Morphological and anatomical comparisons

In the genus *Festuca* there are vegetative characters that are of great taxonomic importance and one of them is the internal morphology of the leaves (Saint-Yves, 1927; Parodi, 1953; Aiken et al., 1985). In Patagonian fescues, the distribution of sclerenchyma on the leaves not only helps to explain the geographic distribution of some of the species but also taxonomically separates the species into different groups (Dubcovsky and Martínez, 1988). *Festuca pallescens* can be distinguished by the presence of sclerenchyma under the abaxial epidermis and in the ribs under the adaxial epidermis, but do not possess girders of sclerenchyma in the main and secondary vascular bundles (Dubcovsky and Martínez, 1988). The morphological analyses allowed us to confirm the taxonomic identity of the studied specimens and, in addition, to detect the phenotypic variability of the species throughout its natural distribution range.

The specimens from ACA population (1) were morpho-anatomically the most different among all populations analysed. Unlike the other populations, plants in ACA presented awnless lemma, double girders of sclerenchyma (adaxial to abaxial) in the main and secondary vascular bundles and ligules with shortest hairs. According to Dubcovsky and Martínez (1988), these characteristics are observed in *F. purpurascens*, *F. argentina*, *F. acanthophylla* var. acanthophylla E. Desv. var. acanthophylla, *F. cirrosa* (Speg.) Parodi, *F. monticola* Phil. and *F. acanthophylla* var. scabriuscula. Within the region of ACA population, *F. pallescens* grows in sympatry with *F. acanthophylla* var. scabriuscula and var. acanthophylla. The specimens of ACA population presented other leaf anatomical traits (e.g., number of vascular bundles and the arrangement of subepidermal sclerenchyma) which are more similar to those typical of these two species than to *F. pallescens*. Similarly, the southernmost populations (BLE (20) and GAI (21)) live in sympatry with *F. gracillima*. Although leaf anatomy in BLE specimens is similar to those of *F. gracillima*, those of GAI are not, because they present abaxial girders in the main and secondary vascular bundles. As previously stated for genetic variability, these results might be evidence of ancient hybridization processes that occurred during the Pleistocene at periglacial refugia both at the northern and southern limits of the distribution range. Different putative glacial refugia have been described within the current distribution range of *F. pallescens*, from the north-west Patagonia (~35° S) to the south of Santa Cruz (~51° S) (Sérsic et al., 2011). Moreover, the distribution range of *F. acanthophylla* and *F. purpurascens* on the north and *F. gracillima* on the south match with some of these areas (Dubcovsky and Martínez, 1998) (Fig. 2). The coexistence of these species with *F. pallescens* in confined proximity and through many recolonization cycles, could have promoted interspecific crossing. Hybridization during prolonged isolation at glacial times was reported for other species around the world (e.g., Consaul et al., 2010; Klein and Kadereit, 2016; Liu et al., 2018; Shepherd et al., 2022) and also in Patagonia (e.g., Tremetsberger et al., 2009; Soliani et al., 2012; Azpilicueta et al., 2014).

A latitudinal pattern in the distribution of genetic variation is frequently found within Patagonian species, both plant and animals, mainly related to historical processes like glaciations (e.g., Bekessy et al., 2002; Marchelli and Gallo, 2006; Azpilicueta et al., 2009, 2014; Arana et al., 2010; Cosacov et al., Lessa et al., 2010; Mathiasen et al., 2010; Sérsic et al., 2011; Soliani et al., 2012; 2015; Vera-Escalona et al., 2012; Baranzelli, 2017; Fasanella et al., 2017; Renny, 2022). The variation detected in our study indicates that *F. pallescens* would follow the same latitudinal pattern. Northern populations shared some traits that differed from those recorded in the southern populations. For example, ACA (1) and HUI (4) populations presented the longest ligules. In addition, the populations of northern Patagonia (ACA, HUI, CLI (5), PIL (7) and JAC (9)) differed from the other populations by presenting significantly shorter hairs on the abaxial surface of leaves. Moreover, ACA, HUI, CLI and JAC populations presented significantly shorter spikelets. Discriminant analyses separated ACA, HUI and CLI populations, leaving the rest of the populations without a defined grouping pattern. From the results of the cluster analysis, it is clear that the ACA population separates from the rest of the populations, which grouped into two main subgroups according to their latitudinal geographical distribution (north and south populations).

CONCLUSIONS

Our results showed a low genetic differentiation among central populations, but distinctiveness at peripheral sites suggesting that hybridization of *F. pallescens* with sympatric species is possible; morphological traits and molecular markers converged on similar outcomes. Low genetic variability is an expected outcome of permanent persistence in situ as suggested by Jakob et al. (2009) for *Hordeum* spp. Pollen and phytoliths records showed evidence of the presence of current steppe species belonging to the Poaceae family during the early Quaternary, reinforcing this possibility (Palazzesi and Barreda, 2012; Palazzesi et al., 2021). In the north, the group of analysed populations shared the same distinct molecular variants but displayed differences at morphological traits. Hybridization with sympatric species at these latitudes may be suggested, and even different stages of speciation can be proposed given the high separation of ACA population from the rest of northern populations. This population is at the limit of the distribution range. A similar situation can be inferred in the southern edge, where populations resemble those of *F. gracillima* both at molecular and morphological characters. Marginal sites usually offer suboptimal conditions for a species; therefore, hybridization can play a key role for adaptation (Španiel and Rešetnik, 2022).

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Fig. 1: a) Geographic distribution of the ITS variants of the evaluated populations of *F. pallescens*. *Festuca pallescens* variants are dyed in ranges of green. Numbers above the branches indicate posterior probability (PP) values. *Pappostipa speciosa* var. *speciosa* and *Poa ligularis* were used as outgroups. **b)** Bayesian tree based on nuclear ITS marker.

Fig. 2: Geographic distribution of the haplotypes detected in *F. pallescens* populations along the entire distribution range. Putative glacial refugia (lowland, peripheral and valley refugia as described by Sérsic et al. (2011)) for different plant species are delimited with blue lines. The box on the right shows the haplotype network. Haplotypes are represented with different sized circles according to their frequencies (HyEc: Hybrid Ecotype; Fgra: *F. gracillima*; Fpur: *F. purpurascens*; Farg: *F. argentina*). Point mutations are shown as black lines and indels as red lines.

Fig. 3: Scatterplot of scores derived from discriminant functions Axis 1 vs. Axis 2 produced by discriminant analysis applied to 30 morphological variables for the twelve populations of *Festuca pallescens* studied. ACA (solid squares), CLI (solid inverted triangles), PIL (solid diamonds), HUI (solid triangles), JAC (solid circles), CRO (open triangles), APE (open inverted triangles), GAI (open diamonds), LBA (open squares), RMA (open circles), FON (open pentagon), BLE (open sandglass).

Fig. 4: Leaf cross-section of specimens stained with safranin showing examples of the different combinations of adaxial and/or abaxial girders of sclerenchyma of vascular bundles. Population names follow those of Supplementary data Table S1. A: adaxial to abaxial girders of sclerenchyma in main vascular bundle. B: adaxial to abaxial girders of sclerenchyma in secondary vascular bundles. C: abaxial girders of sclerenchyma in main vascular bundle. D:

abaxial girders of sclerenchyma in secondary vascular bundles. E: secondary vascular bundles without girders of sclerenchyma. F: main vascular bundle without girders of sclerenchyma.

Fig. 5: Dendrogram resulting from cluster analysis obtained with the morphological data of *Festuca pallescens* populations. The morpho-anatomical results of leaf cross-section (following Figure 4) and molecular variation according to ITS and cpDNA were included in the dendrogram.

Table 1: Geographic locations of the sampling sites of *Festuca pallescens* populations. GenBank accession numbers are included.

Population	**Abbreviation**	**Sample site**	**Latitude (South)**	**Longitude (West)**	**GenBank Accession No.**	
					ITS	***trn*L-F**
1	ACA*	Aguas Calientes, Neuquén, Argentina	36° 41' 2"	70° 36' 37"	OP081817	KX701976
2	LMI	Los Miches, Neuquén, Argentina	37° 6' 36"	70° 49' 3"	OP081817	KX701976
3	HUI*	Huinganco, Neuquén, Argentina	37° 12' 17"	70° 37' 12"	OP081817	KX701976
4	CHO	Chos Malal, Neuquén, Argentina	37° 21' 38"	70° 7' 23"	OP081817	KX701976
5	CLI*	Catan Lil 2, Neuquén, Argentina	39° 45' 40"	70° 37' 7"	KX688222	KX701976 OP081824
6	SRA	San Ramón Ranch, Río Negro, Argentina	41° 10' 32"	70° 59' 1"	KX688222	KX701976
7	PIL*	Pilcaniyeu Experimental Field, Río Negro, Argentina	41° 3' 40"	70° 31' 1"	KX688222	KX701976
8	MON	Montoso, Chubut, Argentina	42° 42' 52"	71° 01' 59"	KX688222	KX701976
9	JAC	Ingeniero Jacobacci, Río Negro, Argentina	41° 55' 9"	69° 12' 58"	KX688222	KX701976 KX701978
10	SOM	Somuncura Plateau, Río Negro, Argentina	41° 25' 1"	66° 58' 1"	KX688222	KX701976
11	YAG	Yague, Chubut, Argentina	42° 57' 0"	71° 12' 0"	KX688222	KX701976 OP081824
12	APE*	Arroyo Pescado, Chubut, Argentina	43° 2' 49"	70° 58' 2"	KX688222	KX701976
13	CRO*	Cronómetro, Chubut, Argentina	43° 14' 19"	71° 4' 54"	OP081820	KX701976
14	GOC	Gobernador Costa, Chubut, Argentina	44° 1' 12"	70° 51' 36"	KX688222	KX701976
15	RPI	Río Pico, Chubut, Argentina	44° 8' 24"	71° 26' 24"	KX688222	KX701976
16	FON*	Fontana, Chubut, Argentina	44° 56' 46"	71° 31' 09"	OP081819	KX701976
17	RMA*	Río Mayo, Chubut, Argentina	45° 28' 12"	69° 49' 48"	KX688222 OP081816	KX701976 OP081821 OP081822 OP081824
18	LBA*	Mte Lago Bs As, Santa Cruz, Argentina	46° 36' 0"	71° 7' 48"	KX688222	KX701976

19	LCO	Laguna Colorada, Santa Cruz, Argentina	51° 40' 15"	69° 53' 58"	KX688222	KX701976
20	BLE*	Bajo la Leona, Santa Cruz, Argentina	51° 31' 48"	69° 42' 4"	KX688222	OP081823
21	GAI*	Guer Aike, Santa Cruz, Argentina	51° 37' 33"	69° 37' 53"	OP081818	OP081825

Figure 1

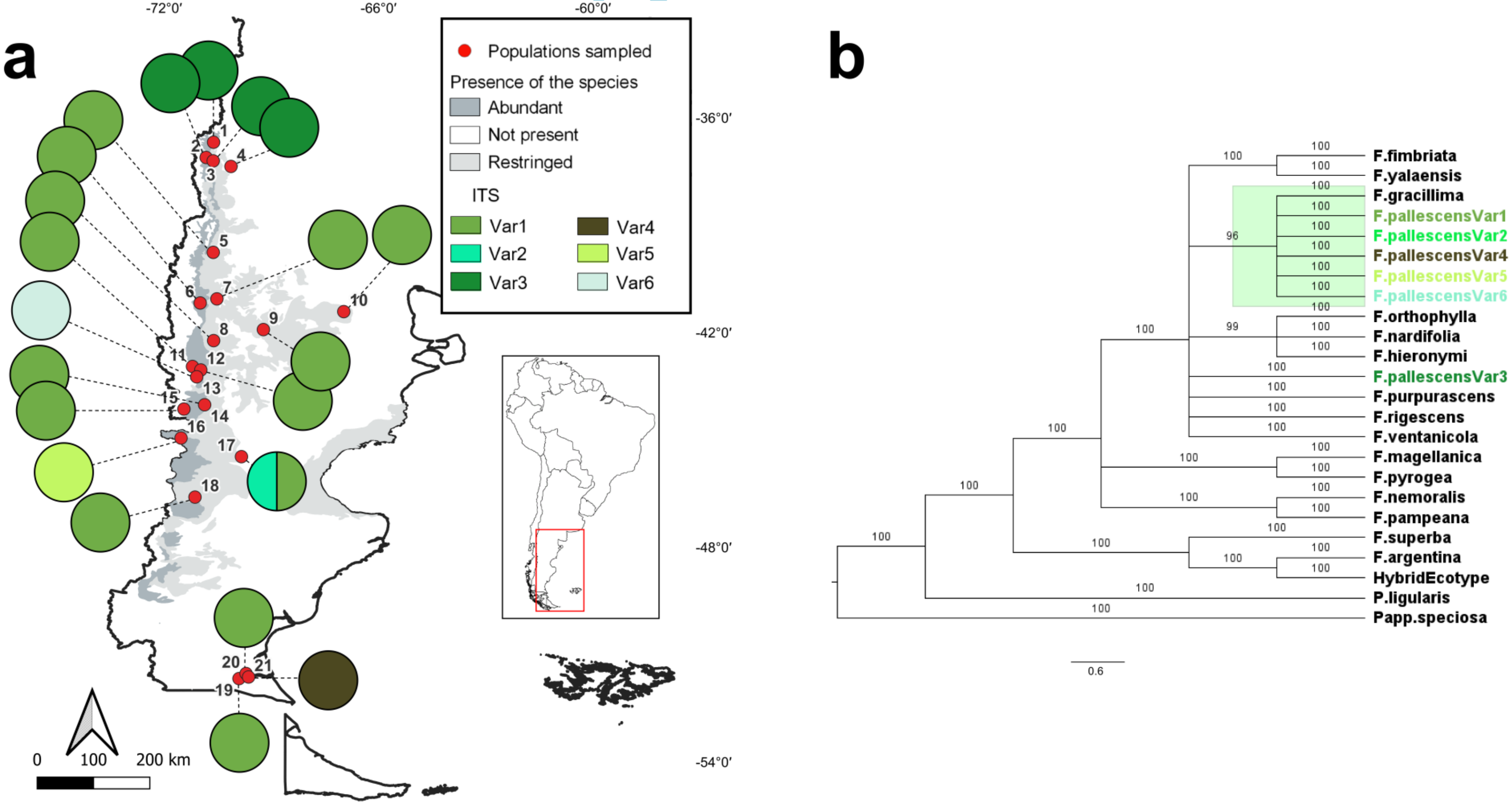
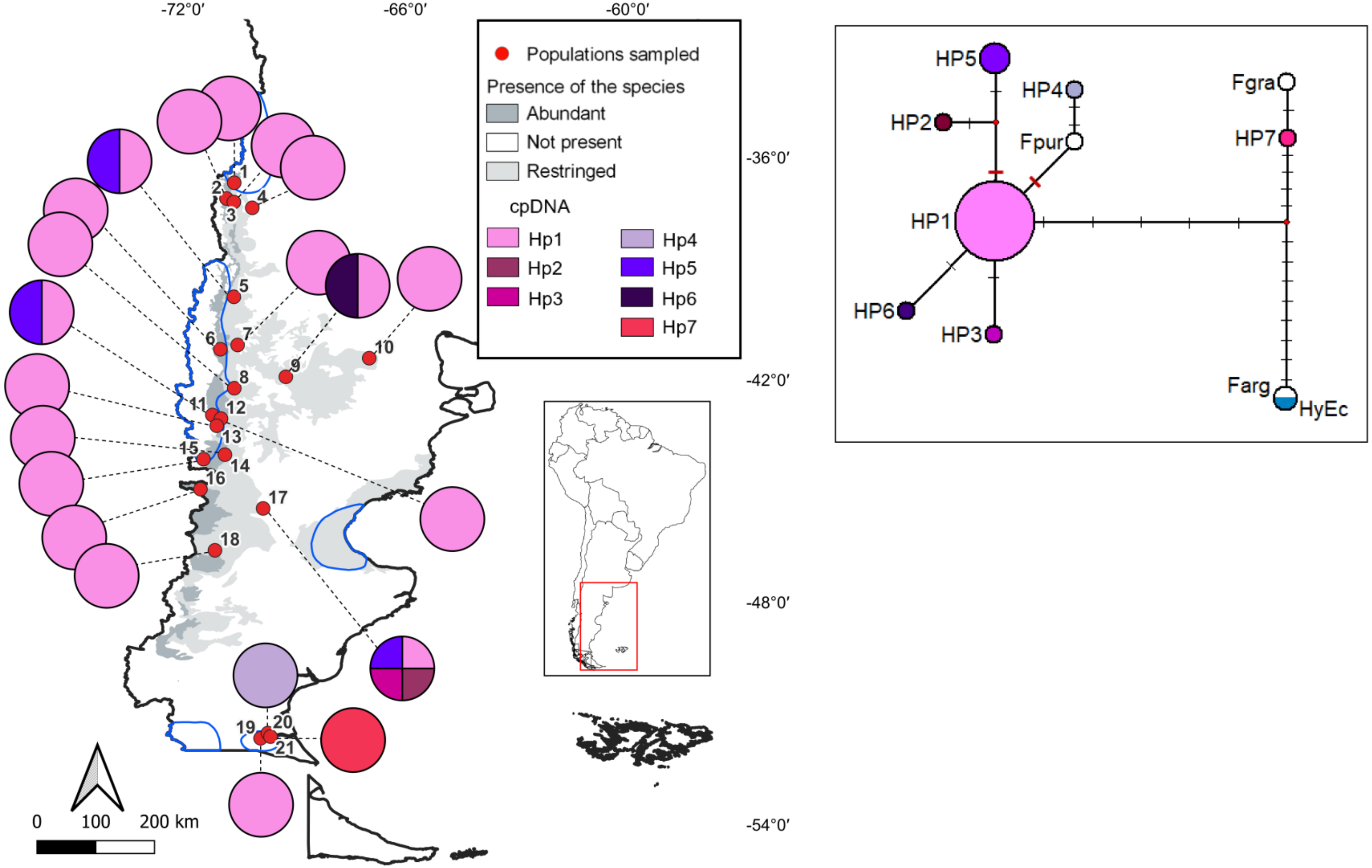

a
-72°0′
-66°0′
-60°0′
-36°0′
-42°0′
-48°0′
-54°0′
● Populations sampled
Presence of the species
Abundant
Not present
Restringed
ITS
Var1
Var2
Var3
Var4
Var5
Var6
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
0
100
200 km
b
F.fimbriata
F.yalaensis
F.gracillima
F.pallescensVar1
F.pallescensVar2
F.pallescensVar4
F.pallescensVar5
F.pallescensVar6
F.orthophylla
F.nardifolia
F.hieronymi
F.pallescensVar3
F.purpurascens
F.rigescens
F.ventanicola
F.magellanica
F.pyrogea
F.nemoralis
F.pampeana
F.superba
F.argentina
HybridEcotype
P.ligularis
Papp.speciosa
100
96
99
0.6

Figure 2


-72°0′
-66°0′
-60°0′
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-48°0′
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1
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9
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11
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13
14
15
16
17
18
19
20
21
0
100
200 km
● Populations sampled
Presence of the species
Abundant
Not present
Restringed
cpDNA
Hp1
Hp2
Hp3
Hp4
Hp5
Hp6
Hp7
HP5
HP4
HP2
Fpur
Fgra
HP7
HP1
HP6
HP3
Farg
HyEc

Figure 3

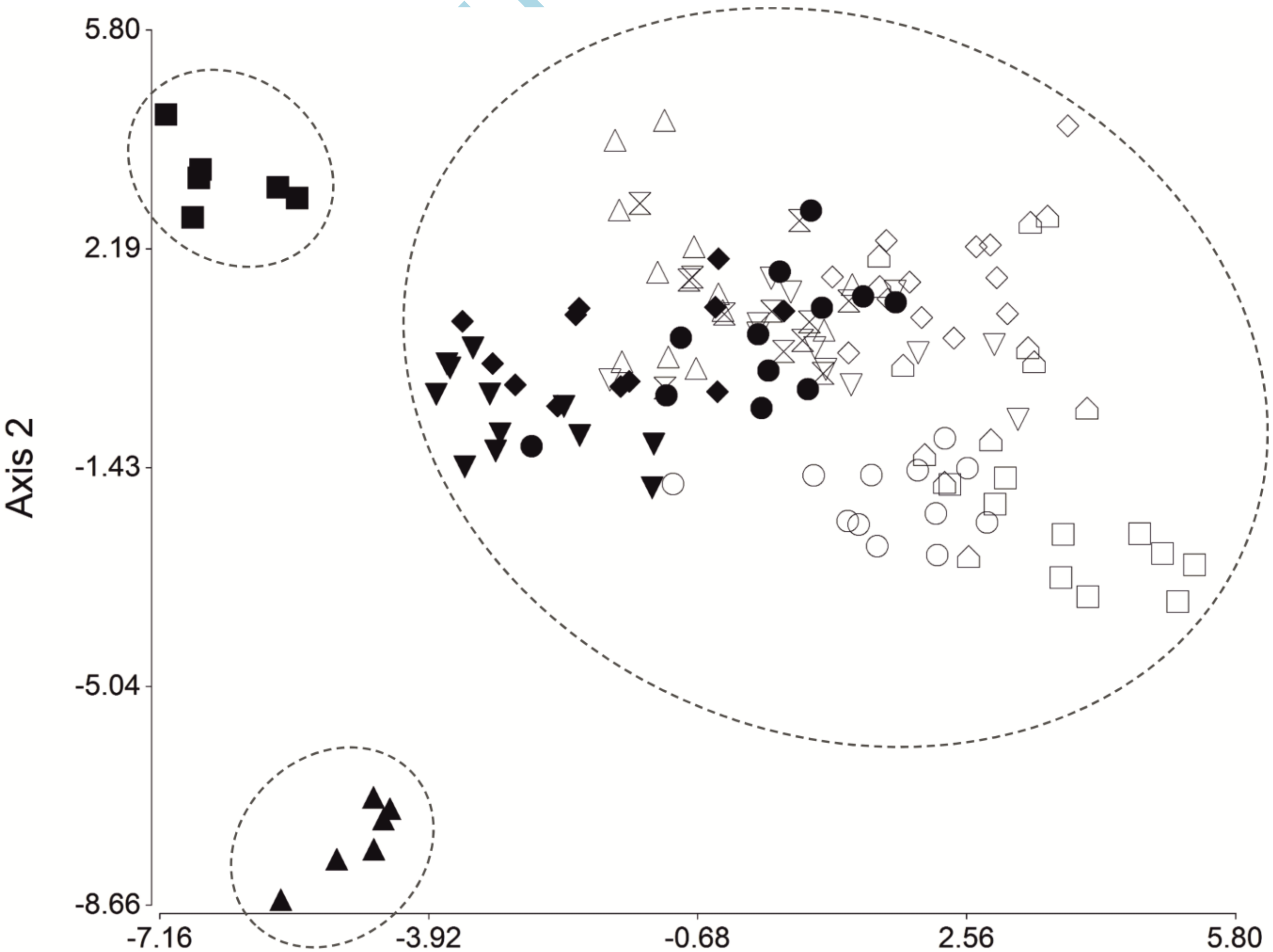


Figure 4

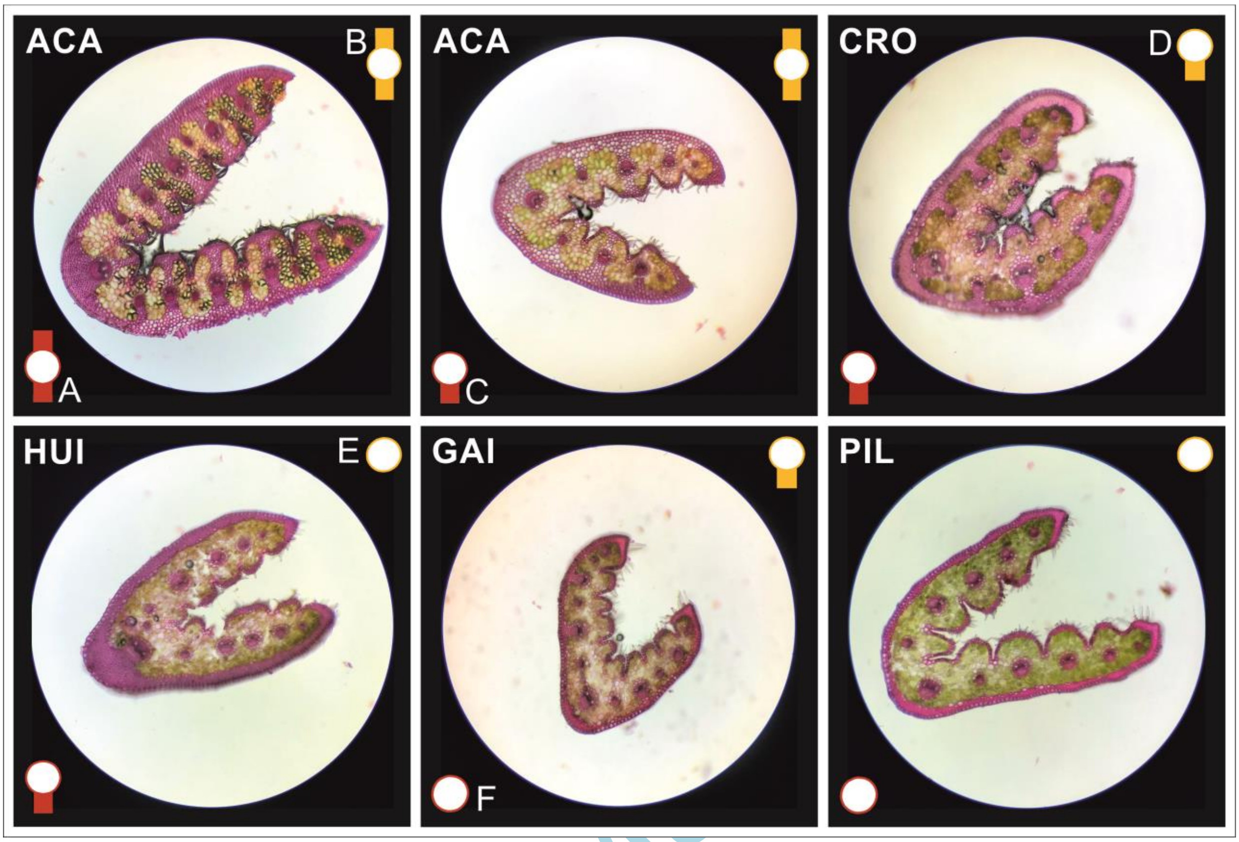
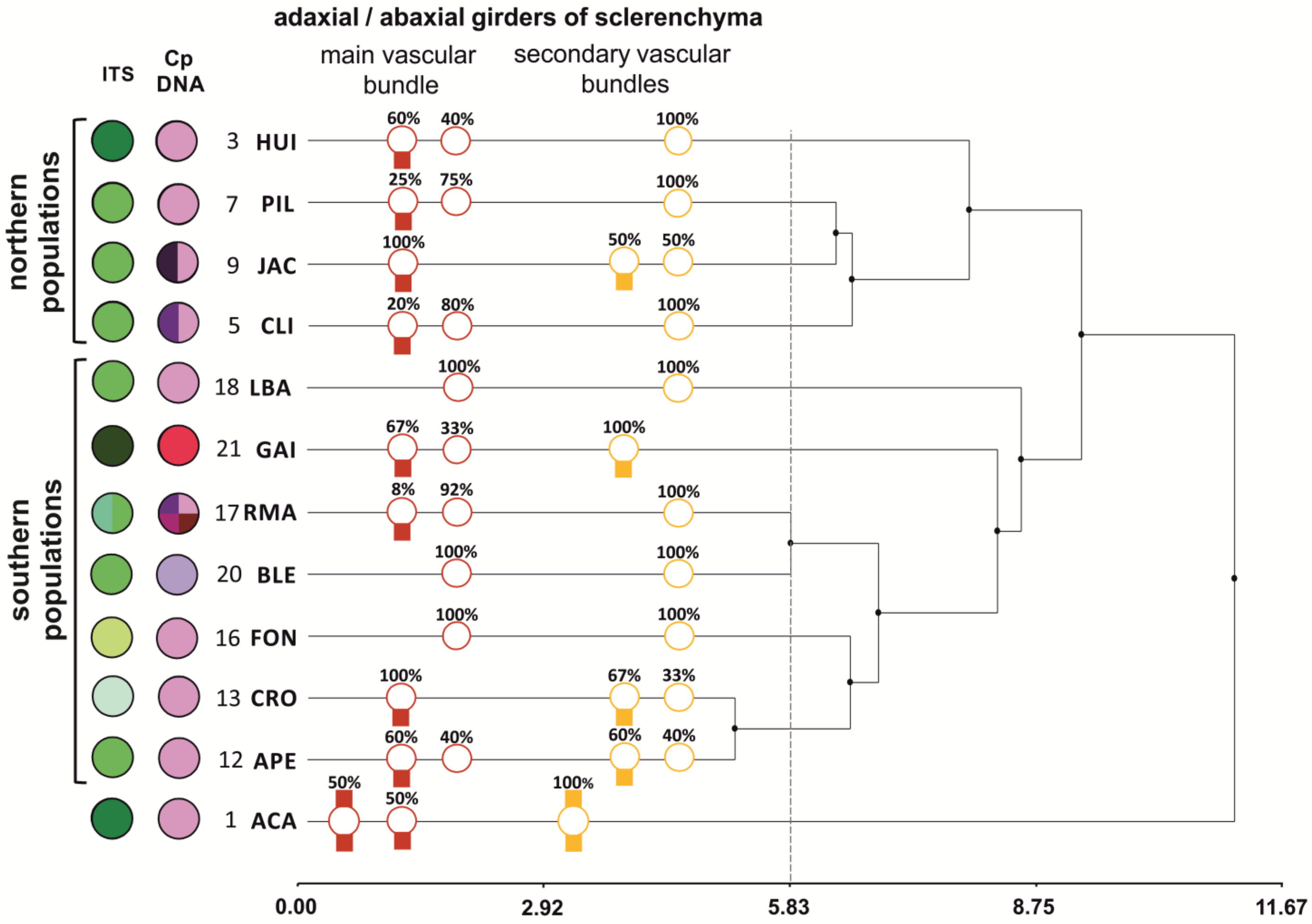


Figure 5


adaxial / abaxial girders of sclerenchyma
main vascular bundle
secondary vascular bundles
ITS
Cp DNA
northern populations
southern populations
3 HUI
60%
40%
100%
7 PIL
25%
75%
100%
9 JAC
100%
50%
50%
5 CLI
20%
80%
100%
18 LBA
100%
100%
21 GAI
67%
33%
100%
17 RMA
8%
92%
100%
20 BLE
100%
100%
16 FON
100%
100%
13 CRO
100%
67%
33%
12 APE
60%
40%
60%
40%
1 ACA
50%
50%
100%
0.00
2.92
5.83
8.75
11.67