

## Genetic diversity patterns of arbuscular mycorrhizal fungi associated with the mycoheterotroph *Arachnitis uniflora* Phil. (Corsiaceae)

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- **Background and Aims** *Arachnitis uniflora* is a mycoheterotrophic plant that exploits arbuscular mycorrhizal fungi of neighbouring plants. We tested *A. uniflora*'s specificity towards fungi across its large latitudinal range, as well as the role of historical events and current environmental, geographical and altitudinal variables on fungal genetic diversity.
- **Methods** *Arachnitis uniflora* mycorrhizas were sampled at 25 sites. Fungal phylogenetic relationships were reconstructed, genetic diversity was calculated and the main divergent lineages were dated. Phylogeographical analysis was performed with the main fungal clade. Fungal diversity correlations with environmental factors were investigated.
- **Key Results** Glomeraceae fungi dominated, with a main clade that likely originated in the Upper Cretaceous and diversified in the Miocene. Two other arbuscular mycorrhizal fungal families not previously known to be targeted by *A. uniflora* were detected rarely and appear to be facultative associations. High genetic diversity, found in Bolivia and both northern and southern Patagonia, was correlated with temperature, rainfall and soil features.
- **Conclusions** Fungal genetic diversity and its distribution can be explained by the ancient evolutionary history of the target fungi and by micro-scale environmental conditions with a geographical mosaic pattern.

**Key words:** Arbuscular mycorrhizal fungi, Andean–Patagonian forest, *Arachnitis uniflora*, genetic diversity, mycoheterotrophy, phylogeography.

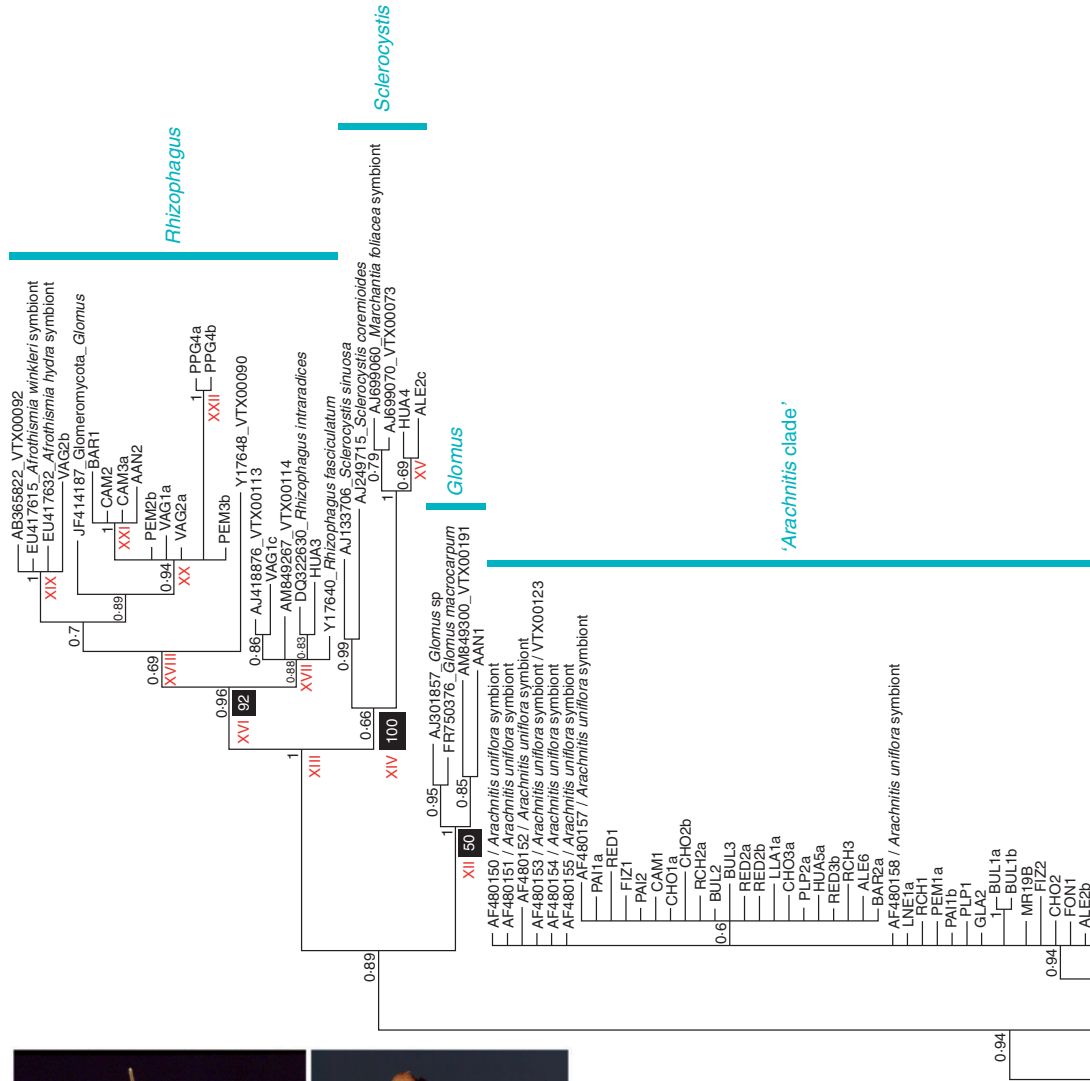
### INTRODUCTION

The most common mycorrhizal symbiosis occurs between arbuscular mycorrhizal fungi (AMF) and plants; in fact, ~70–90 % of land plant species form mycorrhizas with members of the fungal phylum Glomeromycota (Parniske, 2008; Smith and Read, 2008; Merckx *et al.*, 2012). The relationship between AMF and green plants is interpreted as mutualistic, and it is generally assumed that there is bidirectional transfer of nutrients. In this kind of relationship the fungi are obligate partners, because they cannot complete their life cycle without plants. In contrast, arbuscular mycorrhizal green plants are autotrophic and mostly capable of development in the absence of AMF colonization; therefore, plants are considered facultative symbionts in this relationship (Smith and Read, 2008). A third partner, such as an achlorophyllous plant, can interfere with the arbuscular mycorrhizal mutualism; this additional partner takes advantage of that mycorrhizal relationship by becoming a ‘cheater’, which invades a mutualism between two other organisms. Thus, a tripartite symbiosis arises where an obligate cheater plant becomes part of, and depends on the fate of, the mutualism it targets (Bidartondo, 2005; Smith and Read, 2008; Waterman *et al.*, 2013). Achlorophyllous plants sustained by fungi are referred to as mycoheterotrophs (Leake, 1994).

Mycoheterotrophy shows multiple independent origins, and is present in ten families of angiosperms, seven of which are monocots (Merckx *et al.*, 2013a, b).

The biochemical and structural mechanisms of mycoheterotrophic plants to avoid recognition and/or suppress defences in their fungal hosts (*sensu* Merckx *et al.*, 2009) while they sequester fungal carbon are unclear; however, the evolution and maintenance of these pathways may also generate selective pressures towards specialization (Futuyma and Moreno, 1988; Waterman *et al.*, 2013). Furthermore, the dependence of a mycoheterotrophic plant on fungi for its establishment, survival and/or diversification agrees with the observation that some mycoheterotrophic associations are extremely specific (Bidartondo and Bruns, 2002; Bidartondo *et al.*, 2002; Taylor *et al.*, 2002; Leake, 2004). On the other hand, generalists have greater potential to adapt to new environments, providing more opportunities for speciation and a reduced risk of extinction (Zayed *et al.*, 2005). The development of molecular tools shows that not all mycoheterotrophs are extreme specialists; indeed, some mycoheterotrophic plants have the ability to associate with more than one fungal family. Generalist mycoheterotrophic relationships have been found in *Sciaphila ledermannii* (Triuridaceae) which associates with Acaulosporaceae, Gigasporaceae and Glomeraceae, in *Campylosiphon congestus*

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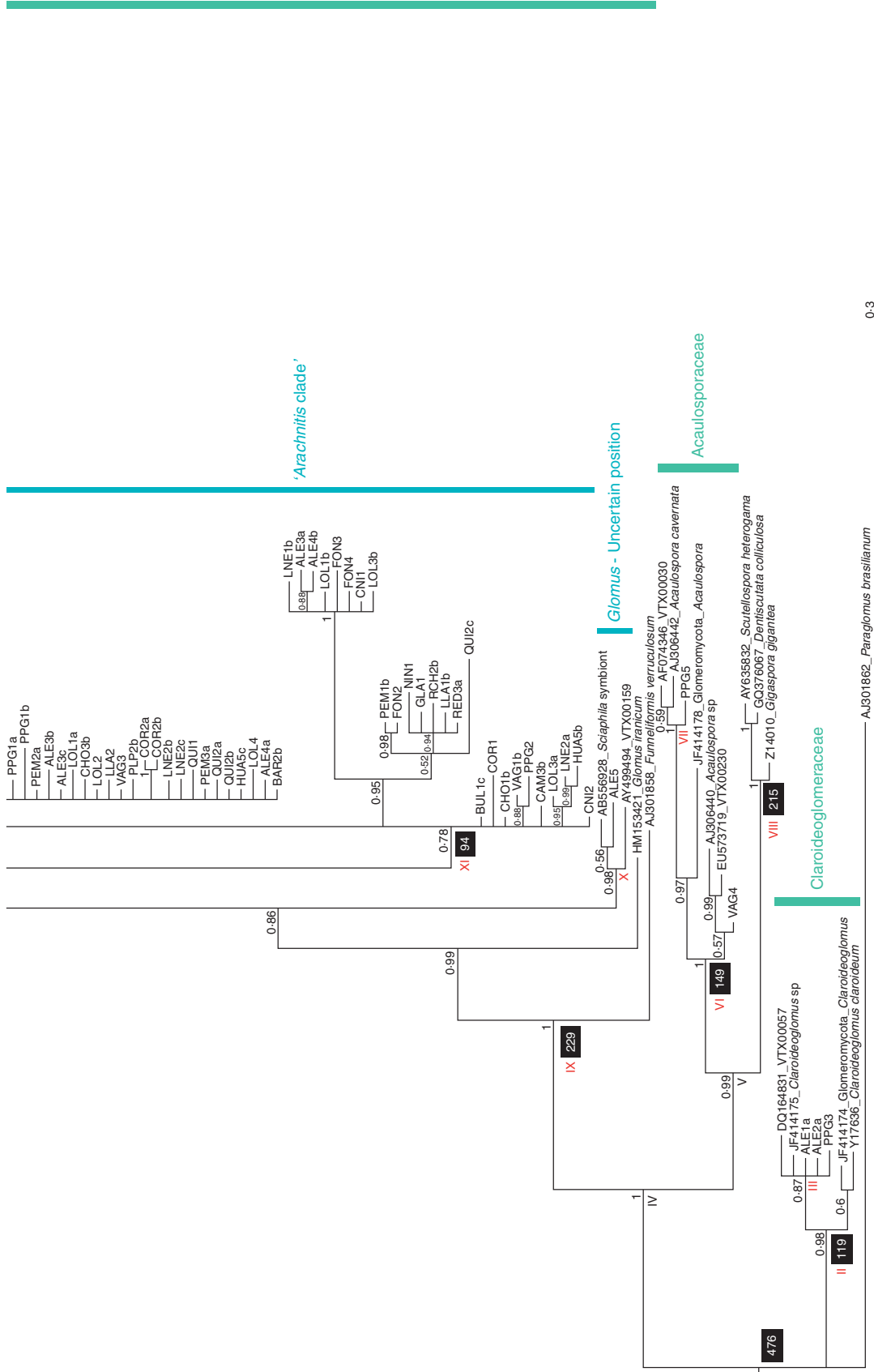


FIG. 1. Bayesian inference tree based on the middle fragment (604 pb) of the nuclear 18S rDNA gene sequences of the glomeromycotan individuals found in *Arachnitis uniflora* roots and other fungal lineages used as identifiers, which are described in Supplementary Data, Table S1. Terminal nodes denoted without GenBank accession numbers correspond to fungi found in this work. Node support is shown as Bayesian posterior probability (BPP) over each node. Nodes with BPP < 0.5 were collapsed to polytomies. Black boxes under nodes indicate main divergence times (in Mya); all Roman numerals correspond to the node ages shown in Table 2, together with their 95% HPD intervals. (Inset) Image of *A. uniflora* plants and details of flower and roots.

(Burmanniaceae) and *Gymnosiphon capitatus* (Burmanniaceae) with Acaulosporaceae and Glomeraceae, and in *Voyria* species (Gentianaceae) with Gigasporaceae and Glomeraceae (Bidartondo et al., 2002; Franke et al., 2006; Merckx et al., 2010, 2012; Courty et al., 2011). Nonetheless, Glomeraceae is the most common family targeted by mycoheterotrophic plants (Merckx et al., 2012), possibly due to its ability to contact roots quickly and to produce an extensive mycelium inside the roots (Hart and Reader, 2002).

There is substantial evidence showing that nutrient availability, dispersal limitation, host plant communities and other environmental factors affect AMF distribution, abundance, root colonization, hyphal development and spore germination (Camargo-Ricalde, 2002; Smith and Read, 2008; Kivlin et al., 2011). In mycoheterotrophic relationships, it is difficult to separate the requirements of each partner, but in general the host fungi delimit the habitat the achlorophyllous plant can occupy (McKendrick et al., 2000; Waterman et al., 2013).

Environmental influence upon the biota is not restricted to the present day. In the last several million years, glaciations and associated climate change, tectonic events, volcanism, palaeobasins, seashore shifts and marine incursions have dramatically altered the landscape, e.g. in South America (Ortiz-Jaureguizar and Cladera, 2006; Ramos and Ghiglione, 2008; Martínez and Kutschker, 2011; Ponce et al., 2011; Sérsic et al., 2011), with concomitant effects on the patterns of distribution and diversification in the biota.

Here, we studied *Arachnitis uniflora* from the completely mycoheterotrophic monocot family Corsiaceae (Fig. 1, inset). This species grows principally in dense and shaded Andean–Patagonian temperate forests of Argentina and Chile, in sub-humid and humid tropical Andean forests in Bolivia, and in the treeless Malvinas–Falkland Islands (Dimitri, 1972; Cribb et al., 1995; Ibsch et al., 1996). Bidartondo et al. (2002) showed a specific association with AMF belonging to *Glomus* group A (currently placed within Glomeraceae) in three populations of *A. uniflora*. It is unclear whether fungal diversity varies as the number of populations increases, especially when including distant areas, as well as different floristic regions. Though evidence indicates that specialization towards a fungal species does not preclude a wide distribution (e.g. the orchid *Eulophia zollingeri*; Ogura-Tsujita and Yukawa, 2008), the extensive geographical range of *A. uniflora* suggests that fungal variation may await discovery due to historical factors and/or adaptation to environmental conditions. To test these expectations, we used fungal DNA sequence data to identify mycorrhizal fungi associated with individuals of *A. uniflora* across nearly all of its geographical range (with the exception of the Malvinas–Falkland Islands). We tested the influence on fungal diversity of current environmental, geographical and altitudinal variables, as well as the role of historical events through molecular dating of fungal clades.

## MATERIALS AND METHODS

### Plant species and sampling

*Arachnitis uniflora* Phil. grows underground and surfaces only during flowering and fruiting, when a shoot ~6–40 cm tall is formed, ending in a single zygomorphic flower (Fig. 1, inset).

The root system consists of a star-like cluster of about ten tuberous roots that store starch and oil and are densely colonized internally by mycorrhizal fungi (Dimitri, 1972; Ibsch et al., 1996; Domínguez and Sérsic, 2004; Domínguez et al., 2005) (Fig. 1, inset). It inhabits shaded forests, where it co-exists with *Austrocedrus chilensis* (Cupressaceae), *Nothofagus* spp. (Nothofagaceae), *Osmorhiza chilensis* (Apiaceae), *Araucaria araucana* (Araucariaceae), *Chusquea culeou* (Poaceae), *Luma apiculata* (Myrtaceae) and *Lomatia hirsuta* (Proteaceae).

One hundred and twenty-three *A. uniflora* were collected from 24 sites along the Andean–Patagonian forests from Argentina and Chile and the Bolivian forests to cover the geographical range of the species (Table 1). The whole root system was removed, washed and immediately stored in 2 % CTAB buffer, and preserved at  $-20^{\circ}\text{C}$  until further processing. All voucher specimens were deposited in the CORD herbarium. All nucleotide sequences from the LAV site (Verde Lake, Villa La Angostura) were obtained by Bidartondo et al. (2002) and were retrieved from GenBank.

### Fungal DNA extraction, amplification and sequencing

Total DNA from one thin section of one root of each *A. uniflora* individual was extracted according to Gardes and Bruns (1993) with a purification step using GeneClean (QBioGene). The whole fungal 18S ribosomal rDNA gene was amplified (JumpStart, Sigma), using the specific primers NS1 (White et al., 1990) and EF3 (Smit et al., 1999). The PCR procedure was 2 min at  $94^{\circ}\text{C}$ ; 34 cycles of 30 s at  $94^{\circ}\text{C}$ , 30 s at  $53^{\circ}\text{C}$  and 1 min 30 s at  $72^{\circ}\text{C}$ ; and final extension for 7 min at  $72^{\circ}\text{C}$ . Because initial DNA sequence screening produced some electropherograms of multiple overlapping copies, cloning was performed using TOPO TA kits (Invitrogen, UK). At least four putative positive colonies from each amplification product were used. The cycling scheme was 7 min at  $94^{\circ}\text{C}$ ; 25 cycles of 30 s at  $94^{\circ}\text{C}$ , 30 s at  $53^{\circ}\text{C}$  and 1 min 30 s at  $72^{\circ}\text{C}$ ; and final extension for 5 min at  $72^{\circ}\text{C}$ . The primers used were NS1/NS3, NS3/NS5 and NS5/EF3 (White et al., 1990), covering the whole 18S gene. Each fragment was sequenced (BigDye, Applied Biosystems) with a 3730 Genetic Analyzer (Applied Biosystems). Two different matrices were constructed, one including the sequences of the 18S (1514 bp) and another containing only the fragment with the highest number of polymorphic sites (*S*). This fragment (604 bp) corresponds to the middle part of the 18S amplified with the NS3/NS5 primers. Sequences were identified by BLAST, and non-glomeromycotan DNA sequences were discarded. Additionally, the sequences were also identified against taxa in the MaarjAM database of Glomeromycota (Öpik et al., 2010).

### Phylogenetic analysis

The fungal sequences obtained from *A. uniflora* roots were assigned by BLAST to virtual taxa (VTX) in the MaarjAM database; those VTX with  $\geq 97\%$  sequence similarity were added to the matrices (ten sequences). Eight *A. uniflora* *Glomus* group A sequences, classified as Glomeraceae by Schüßler and Walker (2010), obtained by Bidartondo et al. (2002), were retrieved from GenBank. Finally, 23 sequences of other genera

TABLE 1. Collection sites and codes (A, Argentina; B, Bolivia; C, Chile), coordinates, altitude, genetic diversity indices (for the obtained *Glomeromycota* sequences and exclusively for *Arachnitis* clade), and haplotypes found in each site, for fungi associated with *Arachnitis uniflora* roots

Site ID	Site	Latitude	Longitude	Altitude	$\pi$ in <i>Glomeromycota</i>	$H$ in the <i>Arachnitis</i> clade	Haplotypes
VAG	Valle Grande (B)	-18.572	-64.043	2387	0.042	1.000	H1, H11
PPG	Pampa Grande (B)	-18.677	-63.917	2378	0.062	0.667	H1, H11
COR	Corel (C)	-35.535	-71.197	487.3	0.009	0.667	H10, H14
QUI	Quillón (C)	-36.668	-72.460	41.2	0.012	0.500	H1, H16
HUA	Hualpén (C)	-36.805	-73.172	17.7	0.037	1.000	H1–H2, H17
AAN	Alto Antuco (C)	-37.369	-71.697	749	0.054	–	–
CNI	Nielol Hill (C)	-38.724	-72.589	217.6	0.025	1.000	H3, H8
LLA	Llancalil (C)	-39.236	-71.636	333	0.011	1.000	H1–H2, H6
RCH	Ruca Choroy Lake (A)	-39.246	-71.189	1241.9	0.010	1.000	H1–H2, H6, H20
LNE	Los Nevados (C)	-39.327	-71.834	400	0.016	0.900	H1, H3, H17–H18
PLP	La Peña (C)	-39.507	-72.582	153.9	0.003	0.667	H1–H2
LOL	Lolog Lake (A)	-40.683	-71.373	936.9	0.019	0.867	H1, H3, H13, H17
LAV <sup>d</sup>	Verde Lake, Villa La Angostura (A)	-40.777	-71.659	813	0.000	0.000	H1
BAR	San Carlos de Bariloche (A)	-41.053	-71.541	822.3	0.015	0.476	H1–H2
PEM	Perito Moreno Hill, El Bolsón (A)	-41.857	-71.537	593.2	0.027	0.500	H1, H4
CHO	Cholila (A)	-42.461	-71.610	580	0.006	0.733	H1–H2, H8
ALE	Los Alerces National Park (A)	-42.887	-71.608	526.2	0.040	0.857	H1–H2, H12, H15, H19
NIN	Los Niños Lagoon (A)	-44.006	-71.490	1026.3	–	–	H6
FON	Fontana Lake (A)	-44.890	-71.527	927.8	0.024	0.833	H1, H3–H4
FIZ	Fitz Roy, El Chaltén (A)	-49.268	-72.950	693.2	0.005	1.000	H1–H2
GLA	Los Glaciares National Park (A)	-50.484	-72.875	403	0.010	1.000	H1, H6
PAI	Torres del Paine National Park (C)	-51.092	-73.198	258.2	0.002	0.667	H1, H2
BUL	Fuerte Bulnes (C)	-53.610	-70.945	403.9	0.006	0.800	H2, H7–H8
CAM	Puerto Cameron (C)	-53.750	-70.095	116.4	0.034	1.000	H8–H9
RED	Redonda Island (A)	-54.863	-68.479	402.2	0.007	0.700	H2, H5–H6

H, haplotype diversity;  $\pi$ , nucleotide diversity.

<sup>d</sup>Only sequences obtained from GenBank (LAV site).

of Glomeraceae cited in Krüger *et al.* (2011) and Redecker *et al.* (2013) were included. *Paraglomerus* (Paraglomeraceae) was selected as outgroup. The complete dataset is shown in Table S1.

Phylogenetic relationships among sequences were reconstructed by Bayesian inference (BI). The GTR + I + G model of DNA evolution was selected under the Akaike information criterion (AIC) as implemented in MrModeltest 2.2 (Nylander, 2004). Analysis was performed in MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) and consisted of two independent runs of  $1 \times 10^7$  generations with four chains (three heated and one cold), sampling every 100 cycles; the first 10 % of the sampled trees (corresponding to the burn-in period) were discarded. Correlation among runs was evaluated in Tracer v.1.6 (Rambaut *et al.*, 2014), considering the effective sample size on each parameter (ESSs; Supplementary Data Table S2). All these procedures were implemented on both matrices (complete and middle part of the 18S gene).

#### Molecular dating

Estimation of the divergence time in the different clades of AMF associated with *A. uniflora* was conducted using three calibration points. The split between Ascomycota, Basidiomycota and Glomeromycota was set to 595 Mya and the crown node of Glomeromycota was constrained to 460 Mya (Redecker *et al.*, 2000). The calibration point to Gigasporaceae was 240 Mya (Padovan *et al.*, 2005). Because the selected priors were located outside the Glomeraceae, it was necessary to

add sequences from Ascomycota, Basidiomycota and representatives of each Glomeromycota family to the alignment used in the above analysis. These sequences were retrieved from GenBank, following Merckx and Bidartondo (2008) and are shown in Table S1. Divergence times were estimated using a Bayesian approach and implementing a relaxed molecular clock model with BEAST v.1.6 (Drummond and Rambaut, 2007). The substitution model was GTR with a Gamma site heterogeneity model with four categories following the MrModeltest result, the clock model was set as an uncorrelated log-normal relaxed model, and the birth–death process was selected as the speciation model. The Monte Carlo Markov chain was set to run for  $2 \times 10^7$  generations, sampling every 1000 cycles; the first 10 % of the sampled trees (corresponding to the burn-in period) were discarded. Correlation among runs was evaluated in Tracer v.1.6 considering the ESSs values on each parameter (Table S2).

#### Genetic diversity and structure

Nucleotide diversity ( $\pi$ ; Nei, 1987) and number of AMF taxa (Glomeromycota families and Glomeraceae genera; see Results section) were calculated at each site, while those sequences belonging to the main AMF clade associated with *A. uniflora* roots (see Results section) were selected to calculate haplotype diversity ( $H$ ; Nei, 1987). Indexes were calculated in DnaSP 5.10 (Rozas *et al.*, 2003). The distributions of these AMF diversities across sites were plotted using the point-to-grid statistical analysis tool in Diva-GIS 7.5.0 (Hijmans *et al.*, 2005). This

plotting was conducted with a grid size of  $0.3 \times 0.3^\circ$  ( $33.3 \times 33.3$  km at the equator) and a circular neighbourhood option of  $1.5^\circ$  (166.5 km).

A haplotype network was built using the median-joining algorithm implemented in Network 5.0 (Bandelt *et al.*, 1999); for this analysis only the sequences grouped in the main AMF clade were selected and autapomorphies and gaps were not considered. Five ambiguous connections (loops) were resolved using the three criteria postulated by the coalescent theory (Crandall and Templeton, 1993).

Patterns of genetic diversity and structure were estimated by genetic landscape shape interpolation analyses using the program Alleles In Space 1.0 (Miller, 2005). This procedure allows the graphical representation of inter-individual genetic distance to detect the location of putative barriers or contact zones with dissimilar gene composition. The analysis was carried out with a grid size of  $50 \times 50$  and a distance weight value of  $a = 1$ . The analysed DNA sequences were those belonging to the main clade of AMF detected, and distributed along Andean-Patagonian range.

The spatial structure of the genetic diversity of the AMF belonging to the main clade associated with *A. uniflora* was analysed across the distribution range using Bayesian inference implemented in Geneland v.4.0.0 (Guillot *et al.*, 2005). Five independent replicate runs were performed, with the number of populations ranging between 1 and 25, assuming a correlated allelic frequency model, and a spatial model without uncertainty on coordinates. Each run consisted of  $8 \times 10^6$  iterations, with a thinning interval of 1000 and a burn-in phase of 800 iterations. Given the complete consistency in the most probable number of populations among runs, the run with the highest posterior probability value was selected, with which membership maps with a  $50 \times 50$  pixel spatial domain were created, according to Guillot *et al.*, 2005.

#### AMF diversity and environmental conditions

The geographical coordinates and altitude of each site were recorded with a GPS. Using the WorldClim database (Hijmans *et al.*, 2005), bioclimatic variables were obtained for each locality at a spatial resolution of  $1 \text{ km}^2$ . From the 19 bioclimatic variables were selected those with biological relevance, trying to avoid correlated variables: annual temperature ( $^\circ\text{C}$ ) and precipitation (mm) averages, temperature and precipitation of the warmest, coldest, driest and wettest quarters (i.e. 3-month seasons; Supplementary Data Table S3). To characterize edaphic features, one soil sample of  $\sim 500$  g was removed from the ground surface to a depth of 15 cm at each location. For each sample, pH, percentages of organic carbon (C), nitrogen (N), sand (Sd) and silt (St), and concentrations of phosphorus (P, ppm) and potassium (K, meq  $100 \text{ g}^{-1}$ ) were determined by the soil laboratory at the Facultad de Ciencias Agrarias (Universidad Nacional de Córdoba). The relationships between AMF nucleotide diversity (Table 1) and environmental factors

(Table S3) were tested using linear regressions with Infostat v.2014 (Di Rienzo *et al.*, 2014).

## RESULTS

Extracted DNA of 123 *A. uniflora* individual roots revealed Glomeromycota sequences in 69 different plants. Sequences of four clones per root retrieved a total of 104 Glomeromycota sequences belonging to 24 sites along the distribution range. Non-Glomeromycota fungi (e.g. Pezizales in Ascomycota and Agaricales in Basidiomycota, principally) were not considered in this study because previous morphological and molecular studies performed on roots of *A. uniflora* never revealed the presence of ectomycorrhizal fungi (Bidartondo *et al.*, 2002; Domínguez and Sérsic, 2004; Domínguez *et al.*, 2009).

#### Phylogenetic analysis

Bayesian phylogenetic relationships were reconstructed with the 104 glomeromycotan DNA sequences, with the addition of the sequences obtained from the GenBank and MaarjAM databases, summarizing 145 sequences (Table S1). Trees were generated with both length matrices; the topologies of both trees were similar (Fig. 1 and Supplementary Data Appendix S1); however, stronger support for each node was found for the tree constructed with the middle part of the 18S gene than for the tree obtained from the entire gene. Thus, further analyses were carried out using only the middle portion.

The selected Bayesian phylogenetic tree (Fig. 1) revealed the presence of fungal symbionts of *A. uniflora* belonging to three well-supported glomeromycotan families, *sensu* Schüßler and Walker (2010): Glomeraceae [Bayesian posterior probability (BPP)=1.00], Acaulosporaceae (BPP=1.00) and Claroideoglomeraceae (BPP=0.98). The Glomeraceae clade was well supported and retrieved the largest portion of the analysed sequences (95.5%), where three genera were recognized: *Glomus* (paraphyletic), *Sclerocystis* (BPP=0.66) and *Rhizophagus* (BPP=0.96); however, 84.1% of these sequences were clustered in an unresolved Glomeraceae group (hereafter termed the 'Arachnitis clade'; BPP=0.78) identified as virtual taxon VTX00123 (*Arachnitis uniflora* symbiont, from Bidartondo *et al.*, 2002) in the MaarjAM database.

The Glomeraceae family clade (Fig. 1) shows an early division separating the genus *Funneliformis* from the remaining genera, which form a large, well-supported clade (BPP=0.99). Most remaining lineages cluster in two clades (BPP=0.86), a small one formed by a *Glomus* of uncertain position (BPP=0.98) and containing one fungus from *A. uniflora* (ALE5) and a large clade with two sub-clades, the *Arachnitis* clade, which is unresolved and contains most *A. uniflora* fungi; and a second clade (BPP=0.89) that also divides in two, one clade containing *Sclerocystis* (BPP=0.66) and *Rhizophagus* (BPP=0.96), and the other with *Glomus* (BPP=1.00) and one fungal sequence from *A. uniflora* (AAN1) roots. The *Sclerocystis* and *Rhizophagus* clades

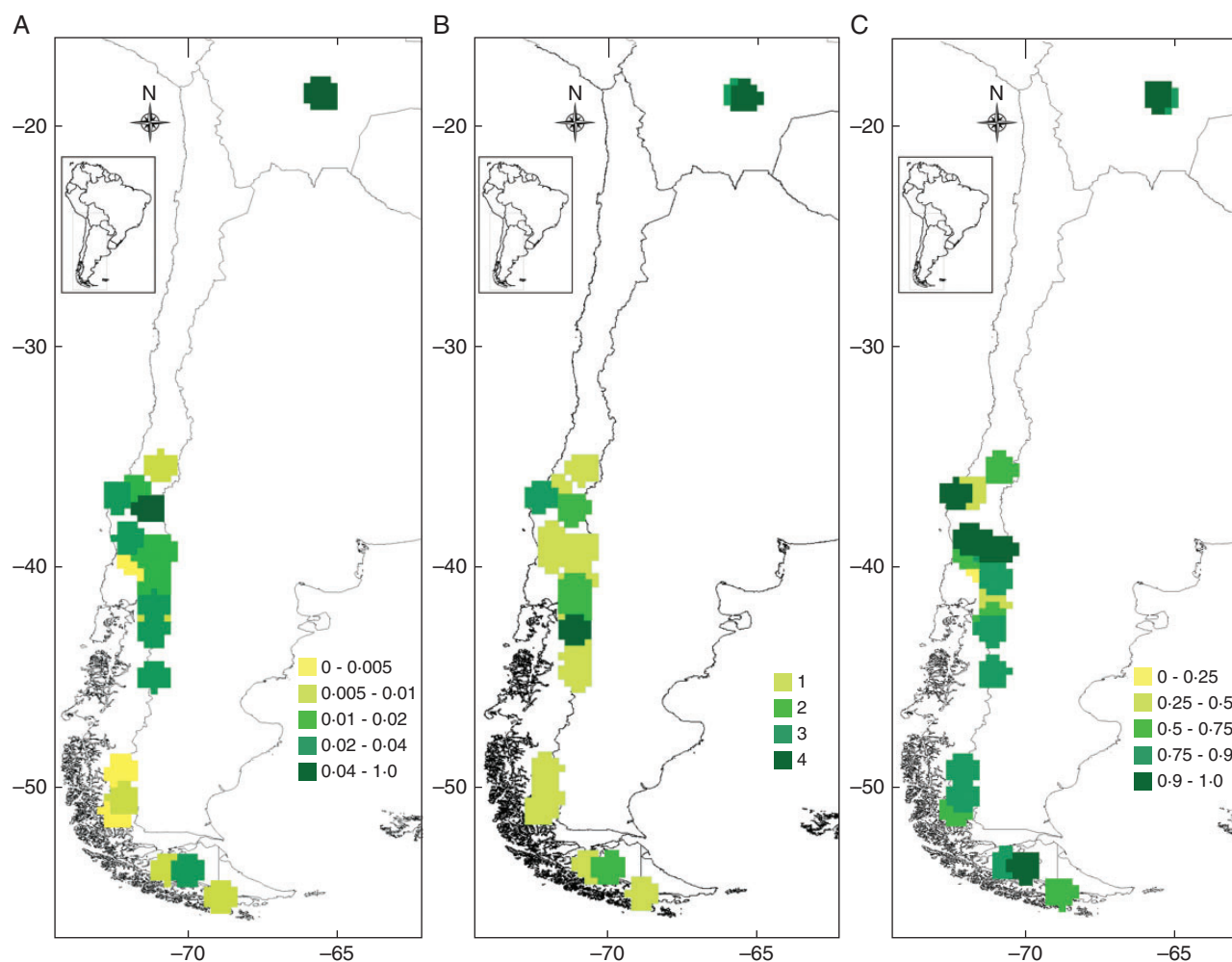


FIG. 2. Geographical distribution patterns of genetic diversity. (A) nucleotide diversity of all *Arachnitis uniflora*-associated fungal tax at each site. (B) Number of glomeromycotan clades per site. (C) Haplotype diversity using only those sequences belonging to the *Arachnitis* clade (Fig. 1). Colour scales indicate genetic diversity values based on a  $33.3 \times 33.3$  km grid cell.

contain two and 13 sequences from *A. uniflora*, respectively. This tree topology suggests that *Glomus* would be paraphyletic.

Outside the Glomeraceae, the families Claroideoglomeraceae and Acaulosporaceae constituted two well supported clades, containing three and two individuals from *A. uniflora*, respectively (Fig. 1). Acaulosporaceae was located within a bigger clade, grouping Diversisporales GenBank sequences (BPP = 0.99). Acaulosporaceae representatives were restricted to the Bolivian forest sites (VAG4 and PPG5). Claroideoglomeraceae fungi were found in one Bolivian site (PPG3) and in two different plants at ALE (ALE1a and ALE2a; Fig. 1, Table 1).

Only two plant individuals presented two fungal families in the same root (Glomeraceae and Claroideoglomeraceae) at ALE, but we detected up to three different AMF clades per root at ALE, CAM, PEM and VAG, among which the *Arachnitis* clade was always present. Within each site, ALE and PPG showed four different Glomeromycota clades, HUA and VAG showed three AMF clades and AAN, BAR, CAM and PEM showed two clades; in the remaining sites only the dominant AMF group, the *Arachnitis* clade, was present. Only at AAN did we find no *Arachnitis* clade representatives (Fig. 1, Table 1).

#### Genetic diversity and structure

Considering the Glomeromycota detected in *A. uniflora* roots, the nucleotide diversity index ( $\pi$ ) showed the highest values for Bolivian and AAN sites, with  $\pi = 0.0617$  in PPG,  $\pi = 0.0545$  in AAN and  $\pi = 0.0416$  in VAG (Table 1). The lowest values were found in LAV ( $\pi = 0$ ). It was not possible to calculate  $\pi$  in NIN because just one fungal sequence was found. In general, AMF genetic diversity tended to increase towards lower latitudes (Fig. 2A); high diversity was detected in Bolivia and the northernmost sites of Chile. However, a cline pattern is not clear due to medium to high genetic diversity detected at the southernmost latitudes. A similar pattern was found when considering the number of different AMF taxa (Acaulosporaceae, Claroideoglomeraceae, *Glomus*, *Sclerocystis*, *Rhizophagus* and *Arachnitis* clade) at each site (Fig. 2B).

Spatial structuring of the genetic diversity of the *Arachnitis* clade evidenced three population clusters (Fig. 3A), according to the BPP. The COR site formed an exclusive cluster (Fig. 3B; BPP = 0.45); Bolivia grouped together with almost all Patagonian sites (i.e. north of COR towards PAI; Fig. 3C; BPP = 0.34); with the exception of the three sites associated

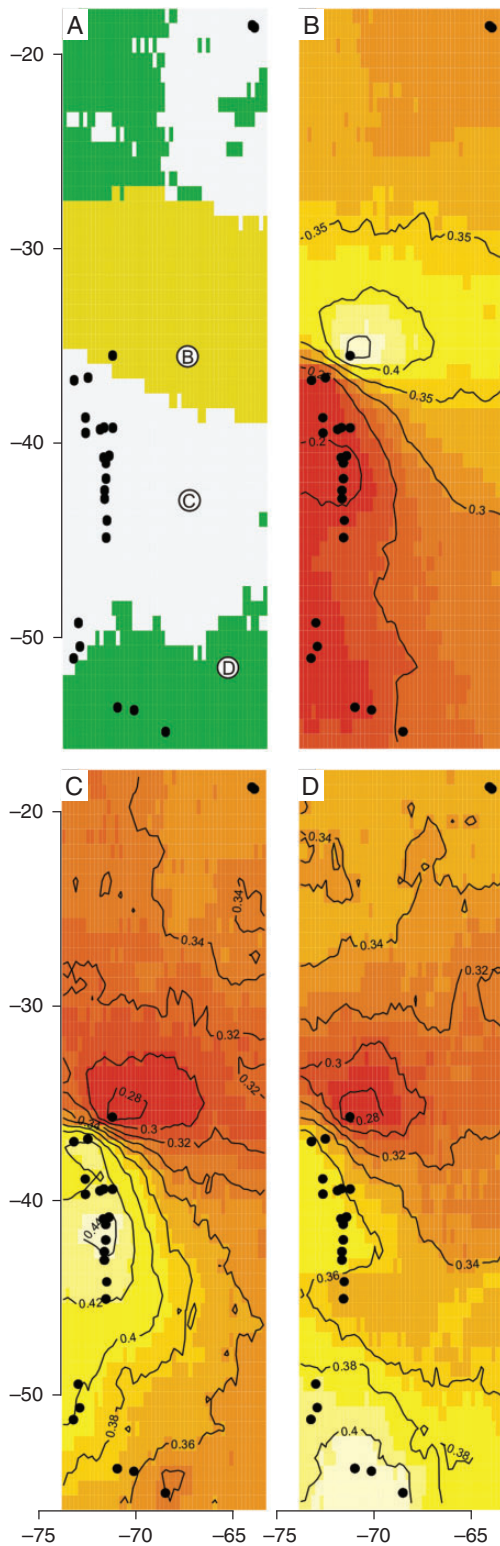


FIG. 3. Bayesian clustering of the AMF belonging to the *Arachnitis* clade. (A) Most probable population membership arrangement. Each colour indicates a cluster. (B–D) Maps of each cluster selected by Geneland; colours indicate the Bayesian posterior probability (BPP), white representing the maximum value and red the lowest. Black dots show each studied site. (B) COR group. (C) Most Patagonian sites and Bolivian representatives. (D) Southernmost Patagonian sites, associated with Tierra del Fuego.

with Tierra del Fuego, which formed the last genetic group (Fig. 3D; BPP = 0.4).

#### *Genetic diversity and phylogeography of the Arachnitis clade*

As the *Arachnitis* clade (i.e. VTX00123) included most DNA sequences and covered nearly the whole geographical range of the plant species, it was used for phylogeographical analyses. Haplotype diversity showed no spatial structure; eight populations showed the highest haplotype diversity values ( $H = 1$ ; Table 1, Fig. 2C), while the lowest values ranging from  $H = 0.69$  to  $H = 0$ . The NIN and AAN sites were not included in the diversity analysis because of scarcity of sequences. The haplotype network displayed a total of 20 haplotypes. Haplotypes H1 and H2 were the most frequent and widespread (Fig. 4); H1 was distributed from the Bolivian sites to PAI, one of the southernmost continental sites, while H2, although absent in Bolivia, extended along the Patagonian forest to Tierra del Fuego. Haplotypes H6 and H8 were also widespread, but less frequent. Considering the mutational steps, H3 and its derivative H15 were the most divergent, separated from H8 by 14 and 15 mutational steps, respectively; these two haplotypes were found between latitudes  $39^\circ$  and  $45^\circ$ S. Haplotypes H4, H6 and H16 formed a group that originated from a non-sampled or extinct ancestor (median vector) and diverged from the rest by three mutational steps. All other haplotypes differed in one or two mutational steps from each other. The most geographically isolated Bolivian sites contained, besides H1, an exclusive haplotype (H11). Haplotypes 5, 7, 9, 10 and 12–20 were exclusive to single populations (Table 1). With the exception of NIN and LAV, all populations were polymorphic, with ALE having five haplotypes, the highest number.

Divergence times among haplotypes from the *Arachnitis* clade were estimated and the phylogenetic tree obtained gave the same network structure, with six main haplotype lineages (Fig. 4C). The origin of this diversification dated from the Upper Cretaceous [93.95 Mya; 95 % highest posterior density (HPD) interval 48.6–158.1 Mya], while the occurrence of the first lineages ranged from 71.2 to 40.9 Mya during the Palaeocene–Eocene, principally, and the last divergences of the current haplotypes occurred during the Miocene (24.2–9.3 Mya). Other important clades of the AMF associated with *A. uniflora* were e.g. Glomeraceae (229.1 Mya; 95 % HPD interval 145.3–315.2 Mya), Claroideoglomeraceae (119.4 Mya; 95 % HPD interval 37.8–241 Mya) and Acaulosporaceae (145 Mya; 95 % HPD interval 59.4–250.1 Mya), and are shown in Fig. 1 and Table 2.

Genetic landscape shape interpolation analyses (Fig. 5) produced surface plots that show two major genetic discontinuities, indicating probable contact areas at  $44^\circ$  around the NIN and FON sites, and another at latitude  $49.4^\circ$ S, between FIZ and GLA, both delimiting a northern and a southern region in the Andean–Patagonian forest. The northern region was in general characterized by the presence of haplotypes derived from H1 (lineage 6; H12, H13, H14, H18 and H19) and haplotypes derived from H8 (lineage 2; H10 and H17 only situated in this region). The most divergent haplotypes, H3 and H15 (lineage 1), were found only between  $37.5^\circ$  and  $46.7^\circ$ S breaks. Although lineage 3 (H4, H6 and H16) was widespread, its haplotypes were



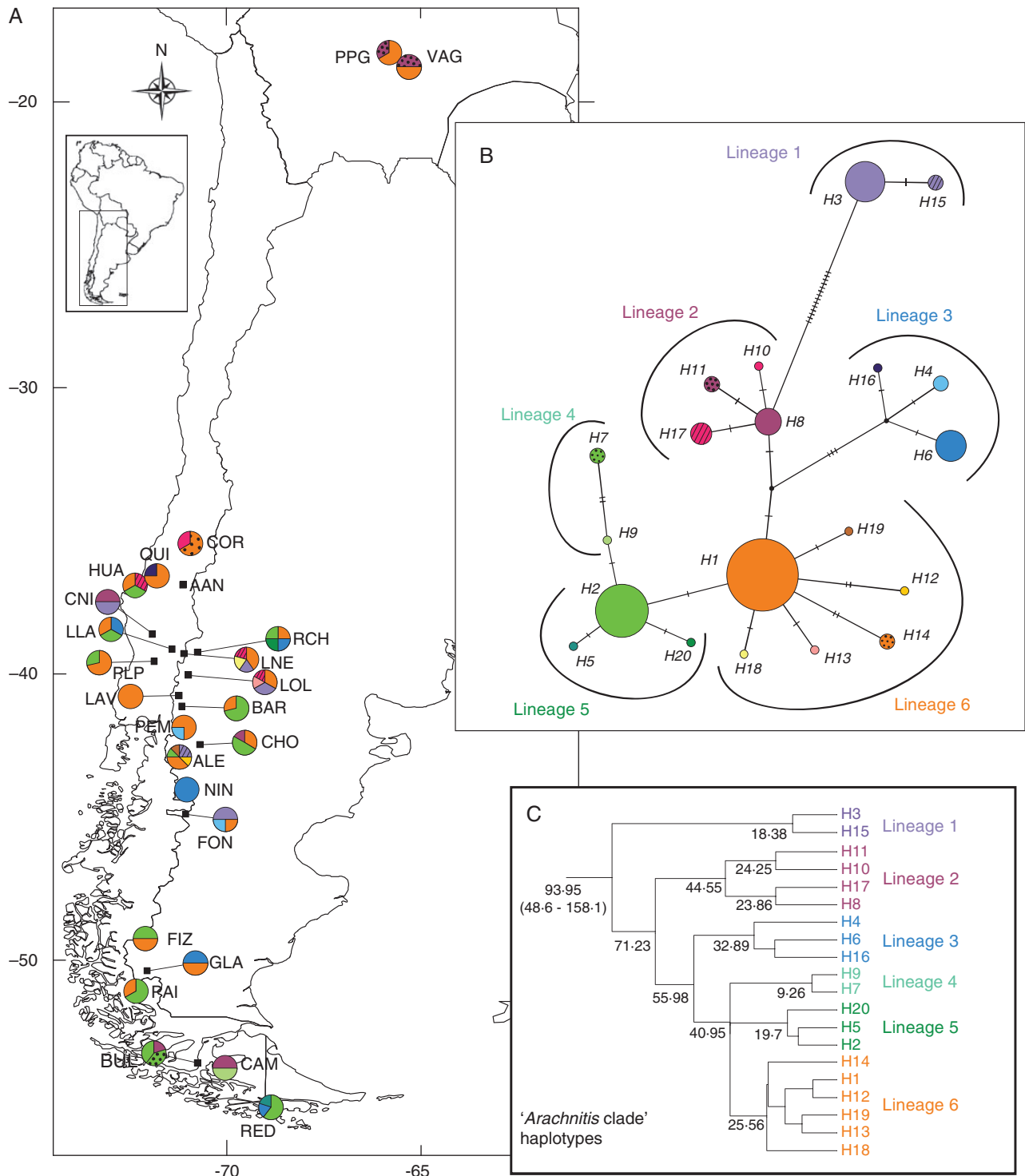


FIG. 4. Geographical distribution and genealogical relationship of the nuclear 18S rDNA haplotypes found in fungi associated with *Arachnitis uniflora* roots and particularly those belonging to the *Arachnitis* clade. Colour correspondence exists between panels. (A) Haplotype distribution. The pie charts reflect the frequency of occurrence of each haplotype in each site. Site codes are referenced in Table 1. (B) Haplotype network. Each short line on the bar between haplotypes represents one mutational step. Black dots represent median vectors and are represented as non-sampled or extinct ancestors. (C) Haplotype lineages found within the *Arachnitis* clade and their respective divergence times (over each node).

TABLE 2. Divergence ages and their confidence intervals (95 % HPD) obtained in the dating of AMF associated with *A. uniflora*

Lineage nodes	Median age	95 % HPD interval
Clades not shown in Fig. 1		
Ascomycota–Basidiomycota–Glomeromycota divergence	595.01	521–667.41
Ascomycota	216.44	55.12–404.42
Basidiomycota	286.53	131.14–488.87
Diversisporales	309.75	245.25–381.73
Glomeromycota clades shown in Fig. 1		
I, Glomeromycota	476.04	420.64–527.5
II, Claroideoglomeraceae	119.39	37.81–241
III	75.62	26.11–153.94
IV	335.01	268.21–406.05
V	279.64	221–346.53
VI, Acaulosporaceae	148.99	59.43–250.14
VII	52.08	17.53–107.53
VIII, Gigasporaceae	215.00	177.12–252.73
IX, Glomeraceae	229.14	145.33–315.18
X, <i>Glomus</i> ; uncertain position	41.67	7.15–105.4
XI, <i>Arachnitis</i> clade	93.95	48.57–158.08
XII, <i>Glomus</i>	49.8	13.61–105.64
XIII	134.56	72.08–214.44
XIV, <i>Sclerocystis</i>	100.16	40.04–173.77
XV	16.78	1.54–51
XVI, <i>Rhizophagus</i>	91.66	44.86–151.32
XVII	46.95	14.48–94.38
XVIII	69.44	32.46–118
XIX	36.97	8.32–82.16
XX	44.75	18.88–82.69
XXI	15.1	3.23–34.59
XXII	2.66	0–14.27

principally distributed in this region, with H4 and H16 only present here. The southern region was dominated by haplotypes derived from H2 (lineages 4 and 5; H5, H7, H9 and H20), being present H1, too. Other smaller peaks also appear at 37.5°, 39° and 46.7°S. Depressions indicate more homogeneous genetic zones, occurring principally around 41.3°S and southwards from 49.4°S.

#### AMF diversity and environmental conditions

Environmental conditions were highly variable across the sampled sites. For example, annual temperature ranged from 4 to 16.8 °C and rainfall from 665 to 2143 mm (Table S3). Temperature of the coldest season and precipitation in the warmest season were the most variable bioclimatic factors, with coefficients of variation (CVs) of 89.6 and 62.5, respectively. In relation to edaphic traits, P concentration was the most variable, ranging from 0.4 to 114.7 ppm (CV=156.2). Linear regressions performed to contrast fungal nucleotide diversity with environmental factors showed some significant associations. Within variables related to temperature, annual temperature (AT) ( $R^2=0.24$ ;  $P=0.0092$ ), mean temperature of the wettest season (TWeS) ( $R^2=0.24$ ;  $P=0.009$ ), mean temperature of the warmest season (TWaS) ( $R^2=0.15$ ;  $P=0.036$ ) and mean temperature of the coldest season (TCS) ( $R^2=0.27$ ;  $P=0.0058$ ) were positively and significantly associated with AMF diversity (Fig. 6A–D). One variable related to precipitation (PDS,  $R^2=0.26$ ,  $P=0.0064$ ) and another to edaphic traits

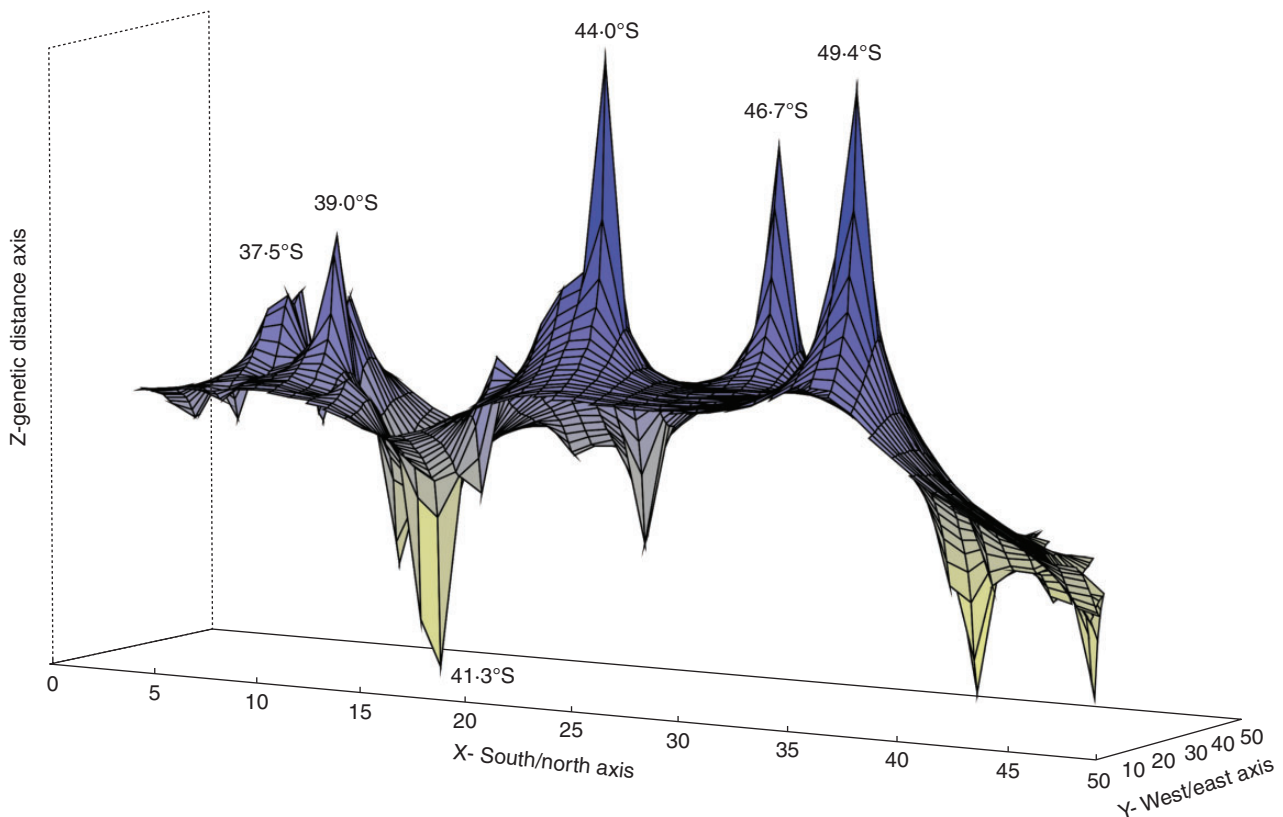


Fig. 5. Genetic landscape shape interpolation analysis using a 50×50 grid size and a distance weighting parameter of  $a=1$ . The  $x$ - and  $y$ -axes correspond to geographic locations; the  $z$ -axis shows genetic distances. Positive peaks show genetic discontinuities or possible barriers to gene flow, and are referenced with latitude coordinates. The analysis was conducted with fungal sequences belonging to the *Arachnitis* clade (Fig. 1).

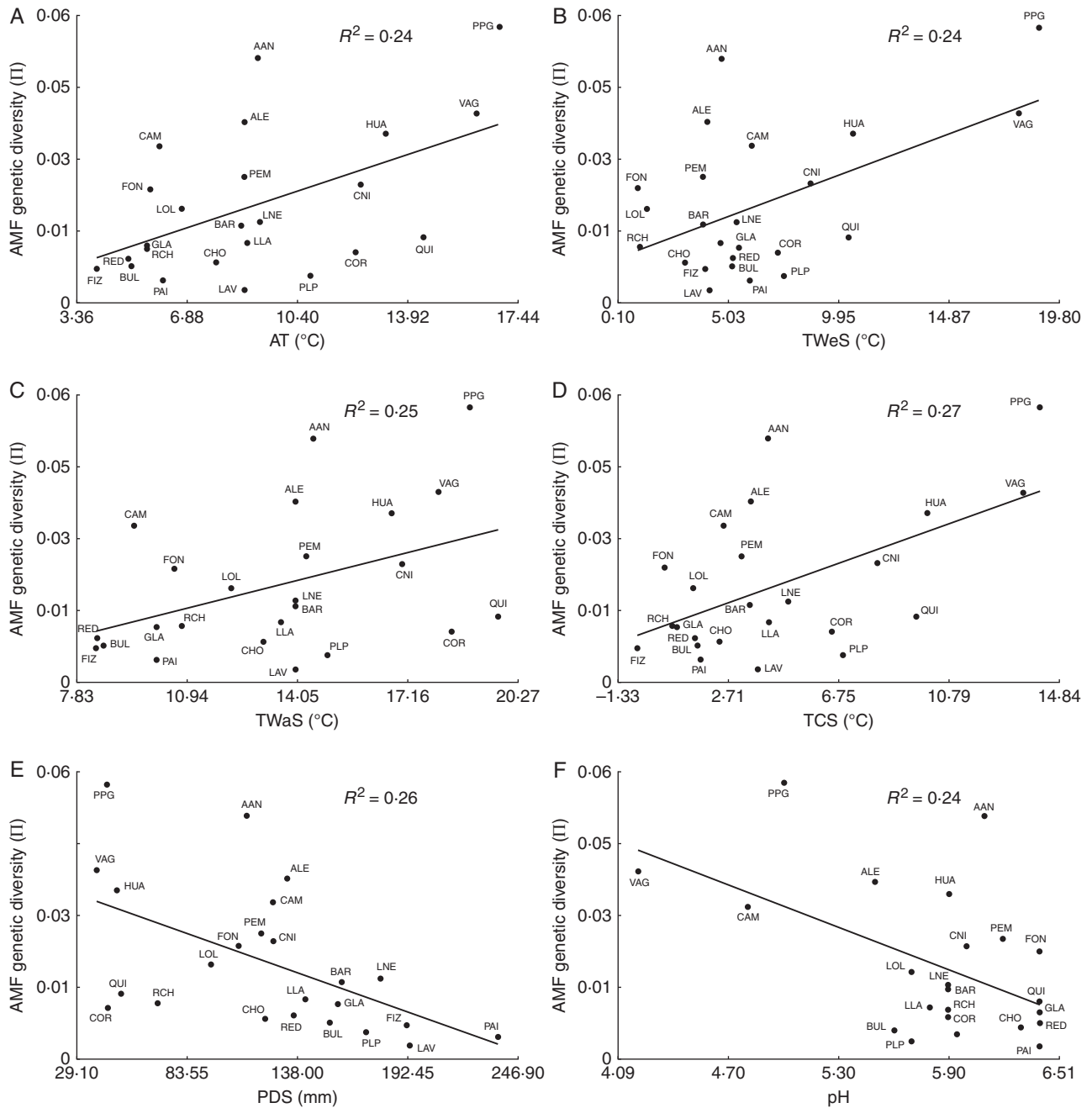


FIG. 6. Relationship between nucleotide diversity of *Arachnitis uniflora* fungi and environmental factors at each site, listed in Table 1. Only significant regressions are shown. (A) Annual temperature. (B) Mean temperature during the wettest season. (C) Mean temperature during the warmest season. (D) Mean temperature during the coldest season. (E) Precipitation during the driest season. (F) Soil pH.

(pH,  $R^2 = 0.24$ ,  $P = 0.0105$ ) were significantly negatively correlated with AMF genetic diversity (Fig. 6E, F). Only significant regressions are shown in Fig. 6.

## DISCUSSION

### Arbuscular mycorrhizal fungi in *A. uniflora* roots

Nearly 95% of the Glomeromycota sequences from *A. uniflora* roots belong to Glomeraceae, supporting conclusions from

Bidartondo *et al.* (2002), who showed that all *A. uniflora* samples were associated with only one AMF lineage, *Glomus* group A. Glomeraceae is the most common AMF in mycoheterotrophic plants (Merckx *et al.*, 2012), and *A. uniflora* is not an exception. However, our study revealed the existence of two additional families associated with *A. uniflora* roots (Acaulosporaceae and Claroideoglomeraceae). The present study and other recent works provide evidence that families other than Glomeraceae can be associated with mycoheterotrophic plants. Acaulosporaceae representatives, as well as the

Glomeraceae genera *Rhizophagus*, *Sclerocyctis* and *Glomus*, were also recorded in other mycoheterotrophic species (Russell and Bulman, 2005; Franke et al., 2006; Merckx and Bidartondo, 2008; Merckx et al., 2010, 2012; Courty et al., 2011; Yamato et al., 2011). However, Claroideoglomeraceae had not been reported before in mycoheterotrophic plants (Franke et al., 2006; Merckx et al., 2012). This is the first study to address the genetic diversity of fungi associated with a mycoheterotrophic plant across its geographical range. In addition to the clade representing most common *A. uniflora* symbionts, the high geographical coverage of this study may have made it possible to recover AMF taxa that associate more rarely with this plant species. These latter may be less effective co-colonizers and/or could represent taxa specific to a certain habitat or geographical region within the range of *A. uniflora*.

The topology of the phylogenetic tree (Fig. 1) is in general congruent with previously published Glomeromycota phylogenetic trees (i.e. Krüger et al., 2011; Redecker et al., 2013). Compared with these, topological differences occurred mainly in the positions of Claroideoglomeraceae (sister family of Glomeraceae) and *Funneliformis* (sister genus of *Glomus*). It is important to note that the topology recovered in the tree constructed with the entire 18S sequence (Appendix S1) was similar to the previously cited phylogenies, but node supports were in general lower, with several non-monophyletic groups. In Fig. 1 *Glomus* appears to be polyphyletic; in fact, this was already observed by Schüßler and Walker (2010), who categorized some *Glomus* individuals and/or clades as ‘species of uncertain position’. Although in these kinds of analysis high phylogenetic resolution is always desirable, our main intention was to test how diverse were the AMF associated with *A. uniflora*.

In the present study, the Bolivian sites showed the highest AMF taxa and genetic diversity, containing representatives of three glomeromycotan families and with Acaulosporaceae exclusively present at those sites. Noticeably, Claroideoglomeraceae was found disjunct in one Bolivian site and in ALE in the Andean–Patagonian region.

As mentioned above, the family Glomeraceae, and essentially the *Arachnitis* clade, are the most common AMF found in *A. uniflora*. This confirms that this plant depends on representatives of Glomeraceae (or the *Arachnitis* clade) for its establishment and/or survival. The other fungal families present more rarely in *A. uniflora* could represent ‘facultative’ mycobionts, as suggested by Franke et al. (2006), or incipient host shifts according to the geographical mosaic theory of coevolution (Thompson, 2005). The latter postulates that variation in species assemblages, in this case that of AMF, would lead to local adaptations, e.g. host shifts. It is important to remark that here we only addressed the AMF part of this symbiosis. With respect to the colonizing ability of the different AMF that makes shifts possible, Hart and Reader (2002) noted that members of Glomeraceae usually contacted roots quickly and produced a more extensive mycelium inside the roots than in soil, while members of the Acaulosporaceae contacted roots more slowly and established a much less extensive mycelium in either roots or soil (Chagnon et al., 2013).

#### Geographical structure of genetic diversity

Considering the geographical distribution patterns of the whole genetic diversity indices (Fig. 2), it was possible to recognize several areas of high diversity that can be hypothesized as glacial refugia for AMF associated with *A. uniflora*, in concordance with sites of high genetic diversity of *A. uniflora* (M. Renny et al., unpubl. res.). The northernmost sites in Bolivia showed high fungal genetic diversity due principally to the presence of fungi of three families and, though sharing H1 with the remaining sites, showed an exclusive haplotype (H11), probably as a response of fragmentation and isolation processes. Across the Patagonian sites, another five putative refugia were identified, such as in the Chilean coastal mountain range at 36·8°S (HUA), also proposed by Sérsic et al. (2011), in the Central Depression of Chile at 38·7°S (CND), where Vergara et al. (2014) found a refugial population of *Nothofagus obliqua* and Acosta et al. (2014) found one *Nothofagus* population with an exclusive haplotype within the same place. It is noteworthy that *Nothofagus* is a common co-occurring genus with *A. uniflora* along its Andean–Patagonian range, and specifically *N. dombeyii* was reported to be associated with *A. uniflora* (Bidartondo et al., 2002). Other refugial areas were located in a longitudinal zone along the western (LNE) and eastern (RCH and LOL) flanks of the Andes between 39·3°S and 40·7°S, at 42·5°–43°S (CHO and ALE), in concordance with refugia proposed in broad analyses by Sérsic et al. (2011) and Souto et al. (2015), and in specific studies by Premoli et al. (2000), Marchelli and Gallo (2004) and Cosacov et al. (2010); while the HUA refuge was proposed only by Sérsic et al. (2011). The southernmost refuge was proposed at 53·7°S (CAM), in agreement with Jakob et al. (2009), Tremetsberger et al. (2009) and Souto et al. (2015). It is remarkable that this last refuge in CAM reinforces the idea of local persistence of the forest in southern latitudes (Premoli et al., 2000). Notably, the appearance of putative co-colonizers or ‘facultative’ fungi coincides with sites VAG, PPG, ALE, HUA and CAM, and zones around these, proposed here as refuges.

Twenty-four of the 25 analysed sites were represented by individuals belonging to the *Arachnitis* clade; this large geographical representation allowed a detailed phylogeographical analysis with this clade. The pattern achieved with the haplotype network and geographical distribution of haplotypes showed no clear geographical structure, evidenced by the wide distribution of the most frequent haplotypes, although it is possible to distinguish two diversification areas: the northern area had a prevalence of haplotypes derived from H1, while the southern area contained mostly haplotypes derived from H2. This is in concordance with the clusters obtained by the statistical test implemented in Geneland.

Centred on the two most frequent and widespread haplotypes (H1 and H2), the network (Fig. 4B) showed a star-like topology – a pattern suggesting a rapid expansion of an ancestral haplotype over a large geographical area (Avise, 2000). Moreover, the four most abundant haplotypes (H1, H2, H6 and H8) were shared in almost all populations, indicating ancient fluid relationships among populations. Thus, geographical distances seem not to be relevant barriers to haplotype connection, as was

already reported by Davison *et al.* (2015); indeed, Bolivian populations shared one out of two haplotypes with the Patagonian forests.

Two main breaks detected with the landscape analyses (interpreted as meeting zones between long-term divergent genetic sources; Fig. 5) at latitudes 44°S and 49.4°S, between sites FON and FIZ, were consistent with the clusters previously mentioned, defining the northern and southern areas. These breaks coincide with a region where no *A. uniflora* records in herbaria or other databases exist; however, we cannot dismiss the possibility that this mainly underground species grows in the area. The presence of a peak at latitude 46.7°S in the middle of the gap suggests that the lack of collections from this area may have affected the surface plot results (Fig. 5). Minor breaks were detected for latitudes 37.5° and 39°S between HUA and CNI, and around LLA, respectively. All latitudinal breaks detected are in agreement with barriers proposed by Sérsic *et al.* (2011) for the Patagonian Andes, except for 46.7°, which was found by Mathiasen and Premoli (2010).

In general, refugia and breaks are correlated with climatic changes associated with Pleistocene glaciations (Sérsic *et al.*, 2011 and references therein), though the molecular dating of haplotype divergences would also show concordance with previous events in the Patagonian Andes. Thus, it was possible to identify an ancient diversification origin within the *Arachnitis* clade during the Upper Cretaceous (93.95 Mya; 95 % HPD 48.6–158.1 Mya). As Corsiaceae diversified more recently, during the Eocene (53–36 Mya; Mennes *et al.*, 2015), it would suggest that these ancient AMF diversifications must have occurred in association with green plants of the community. Fungal diversification and distribution would have enough time to be shaped by successive historic events, beginning in the Cretaceous and continuing until the Pleistocene climate changes. Along the same lines, a study performed on *Nothofagus* (Acosta and Premoli, 2010; Acosta *et al.*, 2014) provides five genetic discontinuities (at 37.5°, 39°, 42°, 40°, 46° and 50°S) in agreement with those found here, which were explained as a result of marine incursions during the Oligocene–Miocene. Finally, the last fungal *Arachnitis* clade haplotype divergences, which occurred during the Miocene, could have occurred in association with diversification events within the Corsiaceae ancestors of *A. uniflora*.

#### Environmental drivers of AMF genetic diversity

Of the three studied environmental factors, temperature (TCS, AT, TWeS and TWaS) was strongly and positively associated with AMF genetic diversity (Fig. 6A–D). Rainfall and edaphic features were poorly correlated with AMF diversity, and were represented only by PDS (from the precipitation variables; Fig. 6E) and pH values (from the edaphic variables; Fig. 6F); both significantly and negatively correlated with genetic diversity. There is knowledge about the influence of several factors – temperature, soil nutrients and characteristics, latitudinal range, and others – on the distribution, richness and fitness of AMF (i.e. Green *et al.*, 1976; Koske, 1987; Porter *et al.*, 1987; Johnson *et al.*, 1991; Allen *et al.*, 1995; Lekberg *et al.*, 2007; Kivlin *et al.*, 2011; Davison *et al.*, 2015; Öpik and

Davison, 2016). Indeed other factors, like the surrounding community or particular AMF species' requirements, can alter the diversity patterns in fungi (Camargo-Ricalde, 2002; Landis *et al.*, 2004; Kivlin *et al.*, 2011).

In line with this close relationship between temperature pattern and fungal diversity in *A. uniflora* roots, Pirozynski (1968) suggested that temperature is the major factor determining the distribution and occurrence of fungi in general. Moreover, Green *et al.* (1976) argued that pH and temperature similarly drive AMF distribution, in agreement with our results (Fig. 6E, F). Temperature has a stimulating effect on AMF (e.g. Schenck *et al.*, 1975; Koske, 1987; Hu *et al.*, 2013) by increasing hyphal P uptake and transport (Gavito *et al.*, 2003). The results here suggest that genetic diversity of AMF positively correlates with pH, as observed in VAG, CAM, PPG and ALE (Fig. 6F, Table S3), which is in agreement with previous findings (Anderson and Liberta, 1992; Coughlan *et al.*, 2000).

Finally, increasing diversity of fungi associated with *A. uniflora* was correlated with sites with low precipitation during the driest season. In the Andean–Patagonian forests, this period coincides with the spring–summer time. In general, AMF colonization peaks are reported in spring and summer seasons (Sigüenza *et al.*, 1996; Lugo *et al.*, 2003; Bohrer *et al.*, 2004) associated with water stress (Kennedy *et al.*, 2002). This driest time was correlated too with the highest spore density periods for different ecosystems (i.e. Mago and Mukerji, 1994; Carvalho *et al.*, 2001; Lugo and Cabello, 2002; Escudero and Mendoza, 2005; Sivakumar, 2013), in line with our findings.

#### Concluding remarks

The non-photosynthetic mycorrhizal plant *A. uniflora* can associate with fungi of three glomeromycotan families, among which Claroideoglomeraceae had never before been found in mycoheterotrophic plants. Glomeraceae fungi of three genera are by far the most common and widespread fungi that nourish this plant species. The *Arachnitis* clade, the main group of AMF with *A. uniflora*, experienced several and successive climatic and geological events that moulded their distribution and diversity. In line with these processes, it is possible that fungi rarely found in this plant may have resulted from facultative associations during adverse environment conditions in several time periods.

In this tripartite relationship, AMF and their chlorophyllous hosts have far more control than mycoheterotrophic plants, considering the huge differences in biomass and because mycoheterotrophic plants depend on AMF and their green hosts, but not vice versa. We found that temperature is the most important factor accompanying the distribution of AMF genetic diversity; however, there was no geographical pattern to temperature. Rather, environment acts at the micro-scale, where some determining factors (e.g. temperature, pH and dry periods acting together or combined differently) create geographical mosaics with particular conditions that allow *A. uniflora* to increase its fungal diversity.

## SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. Table S1: sequences included in the phylogenetic analysis. Table S2: ESS values from Bayesian analyses obtained with Tracer. Table S3: collection sites and IDs, and environmental variables (temperature, precipitation and soil traits) of the sampled sites for fungi associated with *A. uniflora* roots. Appendix S1: Bayesian inference tree based on the complete (1514 pb) nuclear 18S rDNA gene sequences of the glomeromycotan individuals found in *A. uniflora* roots and other fungal lineages used as identifiers, which are described in Table S1. Terminal nodes denoted without GenBank accession numbers correspond to fungi found in this work. Node supports are shown as Bayesian posterior probability (BPP) values at each node. Nodes with BPP < 0.5 were collapsed to polytomies.

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