



Species tree phylogeny, character evolution, and biogeography of the Patagonian genus *Anarthrophyllum* Benth. (Fabaceae)

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Abstract

Geologic events promoting the aridization of southern South America contributed to lineage divergences and species differentiation through geographic (*allopatric divergence*) and biotic and abiotic factors (*ecological divergence*). For the genus *Anarthrophyllum*, which is distributed in arid and semi-arid regions of Patagonia, we assessed how these factors affected species diversification and reconstructed its possible biogeographic history in South American arid environments. Sequences were obtained from two molecular markers: the ITS nuclear region and the *trnS-trnG* plastid region. Using Parsimony, Maximum likelihood and Bayesian inference individual gene trees were reconstructed, and a species tree was obtained using multi-species coalescent analysis. Divergence times among species were estimated using secondary calibrations. Flexible Bayesian models and stochastic character mapping were used to elucidate ancestral geographic distributions and the evolution of the floral and vegetative phenotypes in the genus. Gene trees and species tree analyses strongly support *Anarthrophyllum* as monophyletic; all analyses consistently retrieved three well-supported main clades: High Andean Clade, Patagonian Clade 1, and Patagonian Clade 2. Main diversification events occurred concomitant with the Andean uplift and steppe aridization; the Andean mountain range possibly acted as a species barrier for the High Andean Clade. Vegetative traits showed adaptations to harsh climates in some clades, while pollinator-related floral features were associated with independent diversification in bee- and bird-pollinated clades within both Patagonian Clades. In conclusion, evolutionary and biogeographic history of *Anarthrophyllum* resulted from the action of ecological, historical, and geographic factors that acted either alternatively or simultaneously.

Keywords Arid lands · Andes · Payunia · Molecular dating · Patagonian steppe · Ancestral state reconstruction

Introduction

In evolutionary diversification, concur processes take place at geographic scale, such as the emergence of geographic

barriers, major changes in climatic suitability, and adaptation to ecological factors that act at local scale. Studies on plant diversification often address one of such processes, for instance, adaptation to locally varying pollinator faunas (e.g., Schemske and Bradshaw 1999; Muchhala 2003; Streisfeld and Kohn 2007). Here we analyze to which extent historical events and ecologic factors concur in species diversification of the genus *Anarthrophyllum* (Fabaceae-Genisteae). The species are mainly distributed in the arid and semi-arid Andean and lowland vegetation of Argentina and Chile (Fig. 1) and exhibit marked differences in growth form and flower phenotype. Contrastingly, the monotypic and long thought extinct sister genus *Sellocharis* (*Sellocharis paradoxa* Taub.; Lewis et al. 2005; Conterato et al. 2007; Cosacov et al. 2013) occurs in subtropical areas of southern Brazil (Miotto and Ludtke 2008) suggesting past floristic connections during late Cenozoic climatic changes (Rabassa 2008; Koenen et al. 2013). The first taxonomic treatment of the genus

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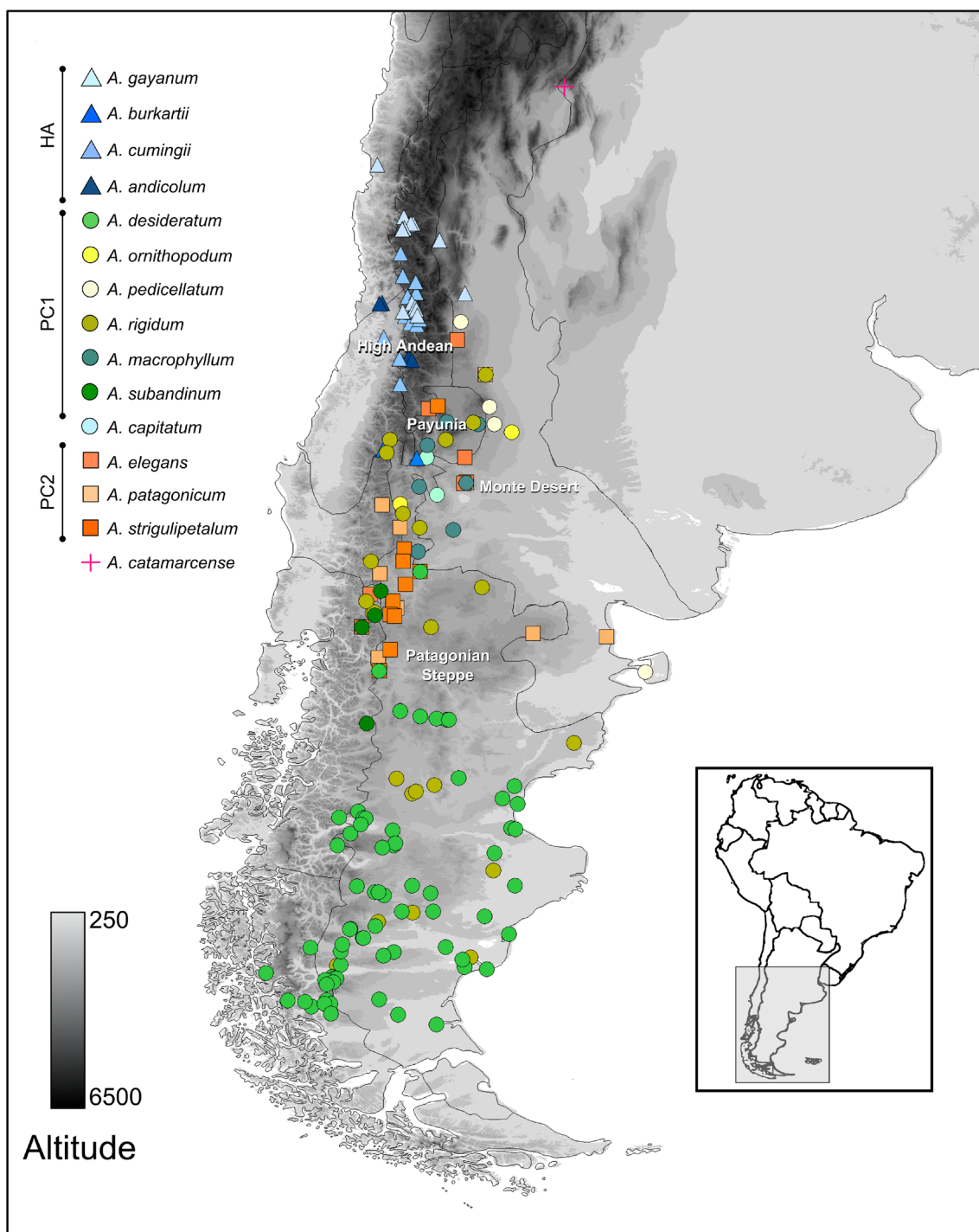


Fig. 1 The inset depicts a map of South America, with the study area indicated by a box. The expanded map shows the distribution of the genus *Anarthrophyllum*. Different species are indicated with different colors. The identified clades are indicated with different shapes: triangle for

species belonging to the High Andean Clade (HA), circles for the species belonging to Patagonian Clade 1 (PC1), and squares for the species belonging to the Patagonian Clade 2 (PC2; see Section 3). The lower left inset indicates altitude (6500–250 m)

Anarthrophyllum identified 15 species based on morphological and anatomical data (Soraru 1974). A previous study (Cosacov et al. 2013) assessed the phylogenetic relationships between five species of the genus using plastid molecular markers.

Hyperaridization of vast areas of South America is attributed to processes that occurred between 15 and 4 Ma (million years ago), most significantly, global climate cooling, the coastal upwelling associated to the establishment of the Humboldt current, and the Andean uplift. This orographic

process began during the Oligocene (around 30 Ma), progressed during the Miocene (17–14 Ma), and reached its maximum at the beginning of the Pliocene (5 Ma; Ortiz-Jaureguizar and Cladera 2006; Ramos and Ghiglione 2008; Hoon et al. 2010). The Andean uplift represented a topographic barrier to atmospheric circulation causing one of the most drastic rain shadows on earth (Houston and Hartley 2003; Blisniuk et al. 2005; Hartley et al. 2005; Hoon et al. 2010). Today in South America, this arid environment extends from tropical to temperate latitudes and from sea level to high elevations occupying a substantial area of the continent (Sarmiento 1975; Cabrera and Willink 1980; Morrone 2006). This area comprising the coastal deserts of Chile and Peru, the Altiplano deserts, and the Monte and Patagonian steppe, which collectively constitute the Arid Diagonal, is characterized by mean annual rainfall lower than 500 mm (Cabrera and Willink 1980; Villagrán and Hinojosa 1997).

During hyperaridization and establishment of the Andean orographic barriers, lineage divergences and species differentiation are expected to result from genetic drift of geographically isolated lineages or populations by reduction of the homogenizing effect of gene flow (*allopatric divergence*) and by biotic and abiotic factors exerting divergent selective pressures under locally varying ecological scenarios (*ecological divergence*). The rapid expansion of newly available habitats during the final Andean uplifts and the emergence of barriers promoting geographic isolation favored the colonization and diversification of several plant lineages in these arid environments (Hughes and Eastwood 2006; Gagnon et al. 2015) such as *Chuquiraga* (Asteraceae; Ezcurra 2002), *Calceolaria* (Calceolariaceae; Cosacov et al. 2009), *Jaborosa* (Solanaceae; Moré et al. 2015), *Lobelioideae* (Campanulaceae; Lagomarsino et al. 2016), *Amicia*, *Coursetia*, *Cyathostegia*, *Mimosa*, and *Poissonia* (Leguminosae; Särkinen et al. 2012). Geographic variation in biotic and abiotic conditions along the Arid diagonal such as contrasting pollinator faunas or levels of aridity likely imposed divergent selective regimes either on key floral reproductive traits (Nosil 2012) or on traits involving plant water balance (e.g., habitat or leaf size; Ezcurra 2002) potentially promoting diversification (see also Ezcurra 2002; Chacón et al. 2012; Cosacov et al. 2009, 2010, 2013, 2014; Martins et al. 2014; Amarilla et al. 2015, Ferreiro et al. 2015; Baranzelli et al. 2014, 2017).

Recent studies have shown that Leguminosae experienced an increase in diversification rates from the Cenozoic onwards associated with the geographic and ecological ranges of certain lineages (Koenen et al. 2013, among others). For the genus *Anarthrophyllum* in particular, the Andes has certainly imposed barriers to its distribution: nine species inhabit exclusively the eastern extra-Andean region across the Patagonian steppe, Monte Desert, and Payunia, a transitional area between the former two (León et al. 1998; Oliva et al. 2001; Roig et al. 2009; Morrone 2015). The remaining group of species inhabits the High Andes (Fig. 1), two species

exclusively on the western slope (Soraru 1974). Within *Anarthrophyllum*, flowers vary markedly in size, shape, color, and position on the branches suggesting adaptations to different functional groups of pollinators (Fig. 2; e.g., Ezcurra 2002; Cosacov et al. 2009; Moré et al. 2015). At least three species are known to be pollinated by finches, other passerines, and one hummingbird species (Paiaro 2010; Paiaro et al. 2012a; Sérsic, personal observation), while other species are reportedly bee-pollinated (Rozzi et al. 1997; Sérsic, personal observation; Fig. 2). The genus is also remarkable for its variation in vegetative phenotype. Though all species are woody, they vary in growth from tall through contracted shrubs to globular cushions with large to minute leaves (Fig. 2).

Based on the geographic distribution and the morphological variation within the genus, we hypothesize, on one hand, that the diversification time of *Anarthrophyllum* is associated to the establishment of the Arid Diagonal during the Andean uplift. On the other hand, that marked divergence in flower and vegetative phenotype (Fig. 2) resulted from *Anarthrophyllum* colonizing habitats with contrasting pollinator faunas and abiotic environments. If the hyperaridization of South America affected *Anarthrophyllum* diversification, we expect to find that main *Anarthrophyllum* diversification events would be associated with main pulses of the Andean uplift; the colonization of new habitats by different lineages would be associated with contrasting environmental conditions, involving changes in the vegetative phenotype and changes in the main pollinator, producing changes in the floral phenotype of the species. To test these predictions, our aims were (1) reconstruct a biogeographic hypothesis for *Anarthrophyllum* based on phylogenetic relationships among the species, geological and geographical events, and ecological scenarios under which diversification of the species occurred; (2) infer when and where the genus originated and diversified; and (3) elucidate ancestral states and the evolution of the floral and vegetative phenotypes in the genus. We feel that the detailed study of this enigmatic and endemic genus of the southernmost arid regions of the world will provide valuable insights toward understanding the evolution of one of the most successful families of flowering plants (the legume family; Lewis et al. 2005).

Material and methods

Taxon sampling and outgroup selection

Sampling included 36 accessions of *Anarthrophyllum* corresponding to the 15 currently recognized species (Soraru 1974). Samples were extracted from dried plant material from herbaria of Argentina and Chile (SI, CONC, CORD, SRFA) and field collections (Table 1). The ingroup database was also complemented with sequences of *Anarthrophyllum cumingii*, *Anarthrophyllum elegans*, and *Anarthrophyllum rigidum*, downloaded from GenBank (Table 1).

Fig. 2 Morphological diversity in *Anarthrophyllum*. Flowers: **a** *A. desideratum*; **b** *A. strigulipetalum*; **c** *A. rigidum*; **d** *A. ornithopodum*; **i** *A. burkartii*. Habits: Shrubs in **e** *A. rigidum* and **j** *A. subandinum*. Globular cushions in **f** *A. strigulipetalum* and **g** *A. desideratum*. **h** Prostrate cushion in *A. gayanum*. **k** Bird pollination in *A. desideratum*. **l** Hymenopterous pollination in *A. ornithopodum*



The outgroup species were selected based on Dormer's (1946) and Lewis et al. (2005) morphological studies that noted similarities between *Anarthrophyllum* and *Sellocharis* (in their fused stipules, appearing to form a sheath around the stem) and on an extensive Legume phylogenetic study performed with ITS and matK sequences that included 235 genera from 37 out of 39 tribes (Wojciechowski et al. 2004). The outgroup species selected for the present study consisted of six representatives of tribe Genisteae where *Anarthrophyllum* belongs: *S. paradoxa* Taub, *Spartium junceum* L., *Genista tinctoria* L., *Laburnum anagyroides* Medik., *Dichilus strictus* E. Mey., *Argyrolobium lunare* L. (Druce), and *Argyrolobium uniflorum* Harv., one species of tribe Crotalariaeae: *Aspalathus longifolia* Benth., and two of tribe Thermopsidaeae: *Baptisia bracteata* Muhl. ex Elliott. and *Thermopsis barbata* Benth. (Wojciechowski et al. 2004; Lavin

et al. 2005; Conterato et al. 2007). Except for *S. paradoxa*, the outgroup sequences were downloaded from GenBank.

DNA extraction, amplification, and sequencing

DNA of freshly collected and herbarium samples was extracted using CTAB method (Doyle 1987). Sequences were obtained from two molecular markers: the ITS nuclear region (nDNA; primers ITS4 and ITS5) and the *trnS-trnG* plastid region (cpDNA; primers *trnS*^{GCU} and *trnG*^{UCC}; Hamilton 1999). These markers were selected since they proved useful for phylogenetic inferences of other legumes for our own previous studies (e.g., Cosacov et al. 2013). The sampling included 31 accessions for the nuclear region and 20 accessions for the plastid region.

Table 1 List of studied specimens of *Anarthrophyllum* and outgroups with data collection, voucher information, GenBank accession numbers, and molecular markers used in this study

Taxon	Country	Province/ Region	Locality	Latitude	Longitude	Elevation	Voucher no./Access no.	nADN	cpADN
<i>A. andicolum</i> (Gillies ex Hook. & Arn.) F. Phil.	Chile	VI Región	Termas del Flaco (P. de Colchagua)	-34.95	-70.4	2230	Baeza, C. 294	x	-
		R. Metropolit- ana	La Domida (P. de Santiago)	-	-	1880	Morrison J.L. 16736	-	-
<i>A. burkartii</i> Soraru.	Argentina	Neuquén	Lag Inferior-Las Ovejas (Dpto. Minas)	-36.853	-70.984	1459	AAC-ANS 4071	x	-
		-	-	-	-	-	SI (not mounted)	x	-
<i>A. capitatum</i> Soraru.	Argentina	-	-	-	-	-	SI (not mounted)	x	x
		-	-	-	-	-	Lavin 769 EF457707	x	-
<i>A. catamarcense</i> Soraru.	Argentina	Catamarca	Andagalá (Dpto. Andagalá)	-	-	-	Schickendantz F. 64	-	-
<i>A. cumingii</i> (Hook. & Arn.) F. Phil.	Chile	R. Metropolit- ana	Provincia de Cordillera	-33.3333	-70.2833	2800	Aedo C. 7089	x	x
			-	-	-	2800	Nicora 4377	-	-
			Provincia de Chacabuco	-	-	-	MAF167695/FJ839486	x	-
<i>A. desideratum</i> (DC.) Benth.	Argentina	Chubut	Tecka-Pampa de Agnia (Dpto. Languineo)	-43.57	-70.565	899	AAC et al. 3514	x	x
			Cruce RP 27-RN 3 (Dpto. Escalante)	-45.507	-67.623	524	AAC et al. 3570	x	x
			El Trebol (Dpto. Escalante)	-45.831	-67.936	524	AAC et al. 3591	x	x
<i>A. elegans</i> (Gillies ex Hook. & Arn.) F. Phil.	Argentina	Mendoza	Portezuelo del Choique (Dpto. Malargüe)	-	-	-	MAF167917/FJ839487	x	-
		Neuquén	Las Lajas (Dpto. Picunches)	-	-	-	Burkart 9749	x	-
			Cerro Lotena (Dpto. Zapala)	-	-	-	Steibel P. et al. 12579	x	x
			Las Lajas (Dpto. Picunches)	-	-	-	Burkart 9749	x	-
<i>A. gayanum</i> (A. Gray) B.D. Jacks.	Argentina	San Juan	Villa Calingasta (Dpto. Calingasta)	-	-	-	Kiesling 8566	x	-
	Chile	R. Metropolit- ana	Provincia de Santiago	-33.33	-70.17	2800	Mihoc M. y Badano E. 137	x	x
<i>A. macrophyllum</i> Soraru.	Argentina	Mendoza	RP 30, Agua Escondida-La Salinilla (Dpto. San Rafael)	-36.1983	-68.5639	1334	AAC-ANS 4447	x	x
			RP 180 (Dpto. Malargüe)	-36.1994	-68.5678	1358	Prina A., Alfonso G., Muiño W. y Morici E. 2532	x	x
<i>A. ornithopodum</i> Sandwith.	Argentina	Chubut	Puerto Piramides (Dpto. Biedma)	-42.565	-64.299	89	AAC-ANS 4480	x	x
			Puerto Piramides (Dpto. Biedma)	-	-	-	Hicken C.M. y Hauman L. 21	-	-
<i>A. patagonicum</i> Speg.	Argentina	Neuquén	RP 13 (Dpto. Picunches)	-38.866	-70.563	1576	Morrone O. et al. 5751	x	-
			Paso Tromen (Dpto. Huiliches)	-	-	-	CORD (not mounted)	x	-
		Río Negro	Cruce RN40-RN 23 (Dpto. Pilcaniyeu)	-40.934	-70.639	986	AAC-ANS 4472	x	x
<i>A. pedicellatum</i> Soraru.	Argentina	La Pampa	Agua Nueva	-	-	-	Troiani, Steibel, Prina 11522	x	x

Table 1 (continued)

Taxon	Country	Province/ Region	Locality	Latitude	Longitude	Elevation	Voucher no./Access no.	nADN	cpADN
<i>A. rigidum</i> (Gillies ex Hook. & Arn.) Hieron.	Argentina	Chubut	Cerro Torres (Dpto. Chical-Có)	–	–	–	Trojani, Steibel, Alfonso s.n.	x	x
			Cruce RP 27-RN 3 (Dpto. Escalante)	–45.507	–67.623	524	AAC-ANS 4075	x	x
			Reserva de La Payunia (Dpto. Malargüe)	–	–	–	MAF163025/FJ839488	x	–
			RN 22 cerca de RP 21 (Dpto. Picunches)	–38.506	–70.484	818	AAC-ANS 4465	x	x
<i>A. strigulipetalum</i> Sorariú.	Argentina	Neuquén	Piedra del Aguila (Dpto. Collón Curá)	–39.9843	–70.0419	660	AAC 3928	x	x
			Río Negro Pilcaniyeu (Dpto. Pilcaniyeu)	–41.1441	–70.6924	900	AAC-ANS 4469	x	x
<i>A. subandinum</i> Speg.	Argentina	Río Negro	S.C. de Bariloche (Dpto. Bariloche)	–41.1095	–71.2157	945	AAC-ANS 4467	x	x
				–41.1095	–71.2157	945	AAC-ANS 4467	x	x
<i>Sellocharis</i> <i>paradoxa</i> Taub.	Brazil	Río Grande del Sur	Viamao: Parque Estatal de Itapua	–30.3584	–51.0241	144	AAC 4519	x	x
				–30.3584	–51.0241	144	AAC 4520	–	x
				–30.3584	–51.0241	144	AAC 4521	–	x
<i>Spartium junceum</i> L.	Gibraltar	Gibraltar	–	–	–	JF338484	x	–	
	United States	California	–	–	–	DQ416808	–	x	
<i>Laburnum</i> <i>anagyroides</i> Medik.	Chile	–	–	–	–	DQ416810	–	x	
	Spain	Madrid	Jardín Botánico de Madrid	–	–	–	AY263679	x	–
<i>Thermopsis</i> <i>barbata</i> Benth.	China	Tibet	–	–	–	David Boufford 105849/AY773353	x	–	
<i>Baptisia bracteata</i> Muhl. ex Elliott	North Ameri- ca	–	–	–	–	AY773349	x	–	
<i>Aspalathus</i> <i>longifolia</i> Benth.	–	–	–	–	–	E. Van Wyk 2799/AM262449	x	–	
<i>Dichilus strictus</i> E. Mey.	–	–	–	–	–	AF287684	x	–	
<i>Genista tinctoria</i> L.	–	–	–	–	–	KX166286	x	–	
	France	–	–	–	–	DQ416807	–	x	
<i>Argyrolobium</i> <i>lunare</i> L. (Druce).	–	–	–	–	–	AF287686	x	–	
<i>Argyrolobium</i> <i>uniflorum</i> Harv.	Tunisia	–	–	–	–	EU341592	–	x	

Amplification for cpDNA consisted of 94 °C for 3 minutes (min), followed by 30 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min. For nDNA, amplification consisted of 95 °C for 2 min, followed by 40 cycles of 95 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min, and finally 72 °C for 8 min. Products were purified using USB Prepease PCR purification plates (Affymetrix Inc., Cleveland, OH, USA), sequenced with BigDye v.3 (Applied Biosystems, Foster City, CA, USA), and purified with Sephadex

(GE Healthcare, Piscataway, NJ, USA) before electrophoresis on an AB 3730xl automated sequencer.

Sequences of each PCR product were checked and edited using ChromasPro v.1.5. For each DNA region, alignment was performed using the ClustalW algorithm (Thompson et al. 1994) implemented in Bioedit v.7.0.9.0 (Hall 1999), using default parameters and adjusted by hand. Gaps were further coded following the simple coding method proposed by Simmons and Ochoterena (2000). In the cpDNA matrix, 11 gaps were coded and in nDNA matrix, 7.

All sequences were deposited in GenBank under accession numbers (MG763562-MG763614).

Phylogenetic analyses: gene trees

Parsimony (MP), Maximum likelihood (ML), and Bayesian inference (BI) analyses were conducted separately for each DNA region. MP analyses were performed using TNT v.1.1 (Goloboff et al. 2008). Heuristic searches were carried out with tree-bisection-reconnection (TBR) branch swapping. All most parsimonious trees were combined in a strict consensus tree using the Nelsen option in TNT. Support was estimated by bootstrap resampling 1000 times (BP_{MP}). Each region was individually tested for best-fit model of sequence evolution using Jmodeltest v.2.1.4 (Darriba et al. 2012), under the Bayesian information criterion. Best-fit substitution models corresponded to GTR + G for ITS and *trnS-trnG*. ML trees were generated using PhyML v.3.0 (Guindon et al. 2005). Support was estimated by bootstrap resampling 1000 times (BP_{ML}). Bayesian inference analyses were conducted using BEAST v.1.7.5 (Drummond and Rambaut 2007). The Markov chain Monte Carlo (MCMC) algorithm was run starting from different random trees. For ITS, two Markov chains were run for 8×10^7 generations, sampling every 8000 generations. For *trnS-trnG*, two Markov chains were run for 1×10^7 generations, sampling trees every 1000 generations. For both DNA partitions, chain convergence was assessed with Tracer v.1.5 (Rambaut and Drummond 2009) to confirm that the estimated sample sizes (ESS) values for all parameters were > 200 . The first 25% of the sampled trees were discarded as burn-in. The remaining trees were used to construct a majority-rule consensus tree and to estimate Bayesian posterior probabilities (PP). Final trees were visualized and edited using FigTree v.1.3.1 (Rambaut and Drummond 2008).

Phylogenetic analyses: species tree and estimates of divergence time

Phylogenetic relationships and support between species were also estimated with BI using the multi-species coalescent module implemented in BEAST 1.7.5 (*BEAST; Heled and Drummond 2010). This method infers a species tree, while taking into account the variation among gene trees. Each locus (ITS and *trnS-trnG*) was treated as a unique partition using best fitting substitution models (GTR + G for both ITS and *trnS-trnG* markers) and a Yule tree prior. Since the standard solution for species tree inference implemented in BEAST (Heled and Drummond 2010) is conditioned on pre-defined terminals, all included specimens were assigned to species according to individual taxonomic assessments (e.g., Terra-Araujo et al. 2015). We used 51 accessions for this estimation (31 for nDNA and 20 for cpDNA). We performed two

independent MCMC runs, each for 10×10^7 generations, sampling trees, and parameter values every 10,000 generations. MCMC runs were combined using LogCombiner v.1.7.5, chain convergence and adequate ESS sampling was assessed with Tracer v.1.5 and the first 25% of the sampled trees were discarded as burn-in. The remaining trees were used to construct a majority-rule consensus tree with TreeAnnotator v.1.7.5.

We also estimated diversification times among *Anarthrophyllum* species on this species tree using *BEAST. As no fossils of *Anarthrophyllum* are known, secondary calibrations were used. Lavin et al. (2005) dated the split between *Anarthrophyllum* and *Spartium* at ca. 19.2 Ma (*Spartium* nests in a clade sister to *Anarthrophyllum* + *Sellocharis* within Genisteeae tribe). Moreover, Cosacov et al. (2013) estimated the divergences between *Anarthrophyllum-Sellocharis* at 17 Ma (between 11.45 and 23.85) and Fountain (2008) estimated the same node at 10.0085 Ma. Based on these, a prior on the age of the node between *Anarthrophyllum-Sellocharis* was set to 14.5 Ma, with a normally distributed standard deviation of 2.5. To improve the estimation, specific mutation rates were used for each DNA partition; for ITS, we used a substitution rate of 2.17×10^{-4} substitutions/site/million years with normal distribution and $DE = 0.000498$. This mean value represents the average between substitution rates of *Ormocarpum* (2×10^{-4} subst/site/Ma; Thulin and Lavin 2001) and *Inga* (2.34×10^{-4} subst/site/Ma; Kay et al. 2006), the two mutations rates known for legume family for this nuclear region. For *trnS-trnG*, we used a uniform prior distribution of mutation rates derived from the analyses of several cpDNA spacers at the intraspecific level with initial value of 7×10^{-4} subst/site/Ma (Palmer 1991) and maximum value of 0.001 and minimum value of 0.0001 (Alsos et al. 2005). The best molecular clock model (strict or relaxed) was evaluated by comparing Model Comparison in Tracer v.1.5, with the relaxed molecular clock model fitting the data best (log10 Bayes factor = 0.5). For each node with strong support, median value and confidence intervals (95% highest posterior density: HPD) were estimated. Finally, trees were visualized and edited using FigTree v.1.3.1.

Ancestral area estimation

To reconstruct the biogeographic pattern of *Anarthrophyllum*, two analyses were performed using the species trees obtained with *BEAST: Bayesian binary MCMC (BBM; Ronquist and Huelsenbeck 2003) and statistical dispersal-vicariance analysis (S-DIVA; Yu et al. 2010) methods in software Reconstruct Ancestral State in Phylogenies (RASPB v2.1; Yu et al. 2015). These methods calculate the optimized areas over a set of trees, thus taking into account topological uncertainty. Based on the current distribution of *Anarthrophyllum* species (Soraru 1974; Paiaro et al. 2012b; Cosacov et al. 2013) and according

to the biogeographic regions proposed by Morrone (2006, 2015), and the ecoregions proposed by León et al. (1998), the distribution areas of each species were defined as follows: (1) Andean region, (2) Monte Desert, (3) Patagonian steppe, and (4) Payunia Desert (Fig. 1 and Table 2). For each species, habitat preferences were compiled from field observations and published data (Soraru 1974; Rozzi et al. 1997) and herbaria records (see supplementary material Table S1). *Sellocharis paradoxa* was excluded from this analysis due to the uncertainty given by the large geographic divergence existing with *Anarthrophyllum*. The input file for RASP consisted of the 1000 post-burn-in trees from the BI analyses and majority-rule consensus tree. Tree files were combined in RASP to estimate Posterior Probabilities for each node. Ten MCMC chains were run simultaneously for 5×10^6 generations and the reconstructed state was sampled every 500 generations. The fixed model Jukes-Cantor was used for BBM analysis with a null root distribution.

Character evolution

In order to reconstruct the evolutionary history of morphological characters in *Anarthrophyllum* accounting for phylogenetic uncertainty and uncertainty in ancestral character states, we implemented two approaches: one for categorical morphological traits and another one for continuous traits (see below). For categorical traits, we conducted stochastic character mappings (Nielsen 2002) using the *make.simmap* function from the R package *phytools* (Revell 2012) for two discrete morphological traits. We performed 1000 stochastic character mapping simulations (SCMS) on the maximum posterior credibility *BEAST tree and plotted the posterior probability of each character state on the corresponding node (Revell 2014). For continuous traits, we used the *heatmap* function from *phytools* for two continuous morphological traits (Revell 2012).

Morphological data were obtained from field observations, herbarium material (supplementary material Table S1), and complemented with data from the literature (Soraru 1974; Paiaro 2010; Paiaro et al. 2012a, 2012b). We selected two categorical and two continuous morphological traits, particularly, floral traits related to pollinator interactions (floral color and floral size) and vegetative traits involved in different strategies for optimal water balance (habit and leaf length). Floral trait measurements and codifications of discrete traits are given in Table 2.

Results

DNA matrices

The nuclear ITS region was 645 nucleotides long of which 94 were parsimony informative (14%). This matrix included 14

of the 15 species of *Anarthrophyllum* and the outgroups *S. paradoxa*, *S. junceum*, *L. anagyroides*, *G. tinctoria*, *D. strictus*, *A. longifolia*, *A. lunare*, *B. bracteata*, and *T. barbata*. The plastid *trnS-trnG* region was 709 nucleotides long of which 78 were parsimony informative (12.1%). This matrix included 12 of the 15 species of *Anarthrophyllum* and the outgroups *S. paradoxa*, *S. junceum*, *L. anagyroides*, *G. tinctoria*, and *A. uniflorum*. All trees calculated with these matrices have been submitted to TreeBASE (<http://purl.org/phylo/treebase/phyloids/study/TB2:S22152>).

Gene trees

Analyses of both ITS and *trnS-trnG* matrices recognized *Anarthrophyllum* as a strongly supported monophyletic group but retrieved different topologies. Parsimony analyses retrieved 8 most parsimonious trees with 346 steps long for the nDNA and 2 most parsimonious trees with 259 steps long for the cpDNA. BI and ML analyses yielded trees with similar topologies to those from the MP analyses but with more strongly supported clades (Fig. 3).

The nuclear ITS tree supported *Anarthrophyllum* as monophyletic ($BP_{MV} = 72$; $PP = 0.95$) and retrieved two major clades (Fig. 3a). Clade A included two subclades: A_1 containing *A. elegans*, *Anarthrophyllum strigulipetalum*, and *Anarthrophyllum patagonicum*, strongly supported ($BP_{MP} = 99$; $BP_{MV} = 99$; $PP = 0.99$) but poorly resolved, and A_2 including *A. cumingii*, *Anarthrophyllum burkartii*, *Anarthrophyllum andicolum*, and *Anarthrophyllum gayanum* ($BP_{MP} = 71$; $BP_{MV} = 80$; $PP = 0.99$). Clade B contained a strongly supported but poorly resolved subclade which included *Anarthrophyllum subandinum*, *Anarthrophyllum desideratum*, *Anarthrophyllum capitatum*, *Anarthrophyllum ornithopodum*, *Anarthrophyllum macrophyllum*, and *Anarthrophyllum pedicellatum* ($BP_{MP} = 72$, $BP_{MV} = 92$, $PP = 0.99$). On the other hand, specimens of *A. rigidum* were recovered together in a strongly supported subclade ($PP = 0.97$; Fig. 3a).

The plastid *trnS-trnG* tree also supported *Anarthrophyllum* as monophyletic ($BP_{MP} = 91$, $BP_{MV} = 96$, $PP = 1.0$) and retrieved two major clades (Fig. 3b). Clade A included two subclades: A_1 clustered the species *A. elegans*, *A. strigulipetalum*, and *A. patagonicum*, with moderate ($BP_{MP} = 80$) to strong support ($PP = 1.0$), and A_2 containing *A. ornithopodum*, *A. pedicellatum*, *A. subandinum*, *A. macrophyllum*, *A. rigidum*, and *A. capitatum* strongly supported only in the Bayesian analyses ($PP = 1.0$). On the other hand, clade B contained two subclades: B_1 clustered the species *A. cumingii* and *A. gayanum* with moderate ($BP_{MP} = 86$, $BP_{MV} = 87$) to strong support ($PP = 1.0$), which was sister to B_2 that contained specimens of *A. desideratum* strongly supported ($BP_{MP} = 99$, $BP_{MV} = 98$, $PP = 1.0$).

Table 2 Biogeographic areas, morphological characters, and pollinators of *Anarthrophyllum* species, used in the ancestral reconstruction

Taxon	Biogeographic region	Floral color	Floral size (mm)	Pollinator	Habit	Leaf size (mm)
<i>A. andicolum</i>	High Andean	Yellow	12.5	?	Creeping cushion	7
<i>A. burkartii</i>	La Payunia	Yellow	14	?	Creeping cushion	6
<i>A. capitatum</i>	La Payunia	Yellow	10	?	Shrub	21.5
<i>A. cumingii</i>	High Andean	Yellow	12.5	Hymenopterans	Creeping cushion	4
<i>A. desideratum</i>	Patagonian Steppe	Red/orange	18	Birds	Globose cushion	35
<i>A. elegans</i>	La Payunia	Red/orange	16.5	?	Globose cushion	20
<i>A. gayanum</i>	High Andean	Yellow	12.5	?	Creeping cushion	8.5
<i>A. macrophyllum</i>	Patagonian Steppe/La Payunia	Yellow	18	Hymenopterans	Shrub	35
<i>A. ornithopodum</i>	La Payunia	Yellow	20	Hymenopterans	Shrub	25
<i>A. patagonicum</i>	Patagonian Steppe/Monte Desert	Red/orange	13.5	?	Globose cushion	11.5
<i>A. pedicellatum</i>	Monte Desert	Yellow	14	?	Shrub	25
<i>A. rigidum</i>	Patagonian Steppe/La Payunia	Yellow	9.5	Hymenopterans	Shrub	12.5
<i>A. strigulipetalum</i>	Patagonian Steppe	Red/orange	17	Birds	Globose cushion	21.5
<i>A. subandinum</i>	Patagonian Steppe	Yellow	13	Hymenopterans	Shrub	8.5

Species tree and estimates of divergence time

The species tree analyses strongly supported *Anarthrophyllum* as monophyletic (PP = 0.99) and recovered two major clades (Fig. 4). The first clade, here labeled “High Andean Clade,” included the species *A. gayanum*, *A. burkartii*, *A. cumingii*, and *A. andicolum* with high support (PP = 0.95). The remaining species were grouped in a weakly supported clade (PP = 0.85) that was further subdivided in two strongly supported clades: “Patagonian Clade 1” (PP = 1.0) which included *A. desideratum*, *A. ornithopodum*, *A. pedicellatum*, *A. rigidum*, *A. macrophyllum*, *A. subandinum*, and *A. capitatum* and “Patagonian Clade 2” (PP = 1.0) contained *A. elegans*, *A. patagonicum*, and *A. strigulipetalum* (Fig. 4).

The median divergence time of *Anarthrophyllum* from its sister species *S. paradoxa* was estimated to be 17.58 Ma (95% HPD = 21.9–13.7) during the early Miocene. The crown age of *Anarthrophyllum* was estimated to be 13.01 Ma (95% HPD = 15.7–8.7) during the Middle Miocene. Divergence time of the most recent common ancestor of the “High Andean Clade” was estimated at 7.5 Ma (95% HPD = 12.7–3.0), during the Late Miocene. The diversification of the “Patagonian Clade 1” was estimated to have occurred 6.47 Ma (95% HPD = 11.2–3.4). Speciation events between 2.6 Ma and 0.5 Ma gave rise to the remaining species of this clade. Finally, the divergence time of the most recent common ancestor of “Patagonian Clade 2” was estimated in 4.18 Ma (95% HPD = 5–0.6) during the Early Pliocene (Fig. 4).

Ancestral area estimation

The most recent common ancestor (MRCA) for *Anarthrophyllum* probably inhabited the Andean region (PP = 73%). The ancestral distribution of the High Andean Clade also was estimated in the Andean region (PP = 99%), whereas the MRCA of Patagonian Clades 1 and 2 may have had a distribution in the Patagonian steppe ($P = 92%$ and $P = 91%$, respectively; Fig. 4). Vicariance was inferred at the node that separated the High Andean Clade from the Patagonian Clades and within the High Andean Clade, whereas dispersal events along the Patagonian steppe, Monte Desert, and Payunia were inferred along the branch of the Patagonian Clades 1 and 2 (Fig. 4).

Ancestral character state reconstructions

The evolutionary pattern of selected morphological traits is summarized in Fig. 5. The ancestor of the genus was inferred to have had a prostrate cushion habit (63% SCMS), with intermediate leaf size (37% SCMS) and medium size yellow flowers (73% SCMS), similar to the ancestor of the Patagonian Clade 1 (Fig. 5). The putative ancestor of the High Andean Clade was inferred to have possessed a prostrate cushion habit (91% SCMS), with small leaf size and short yellow flowers (Fig. 5). In contrast, for the Patagonian Clade 2, the most plausible ancestral condition was a globular cushion (100% SCMS), with intermediate leaf size and large red-orange flowers (100% SCMS; Fig. 5). The stochastic character mapping suggests that larger red-orange flowers and the globular cushion habit evolved independently at least two

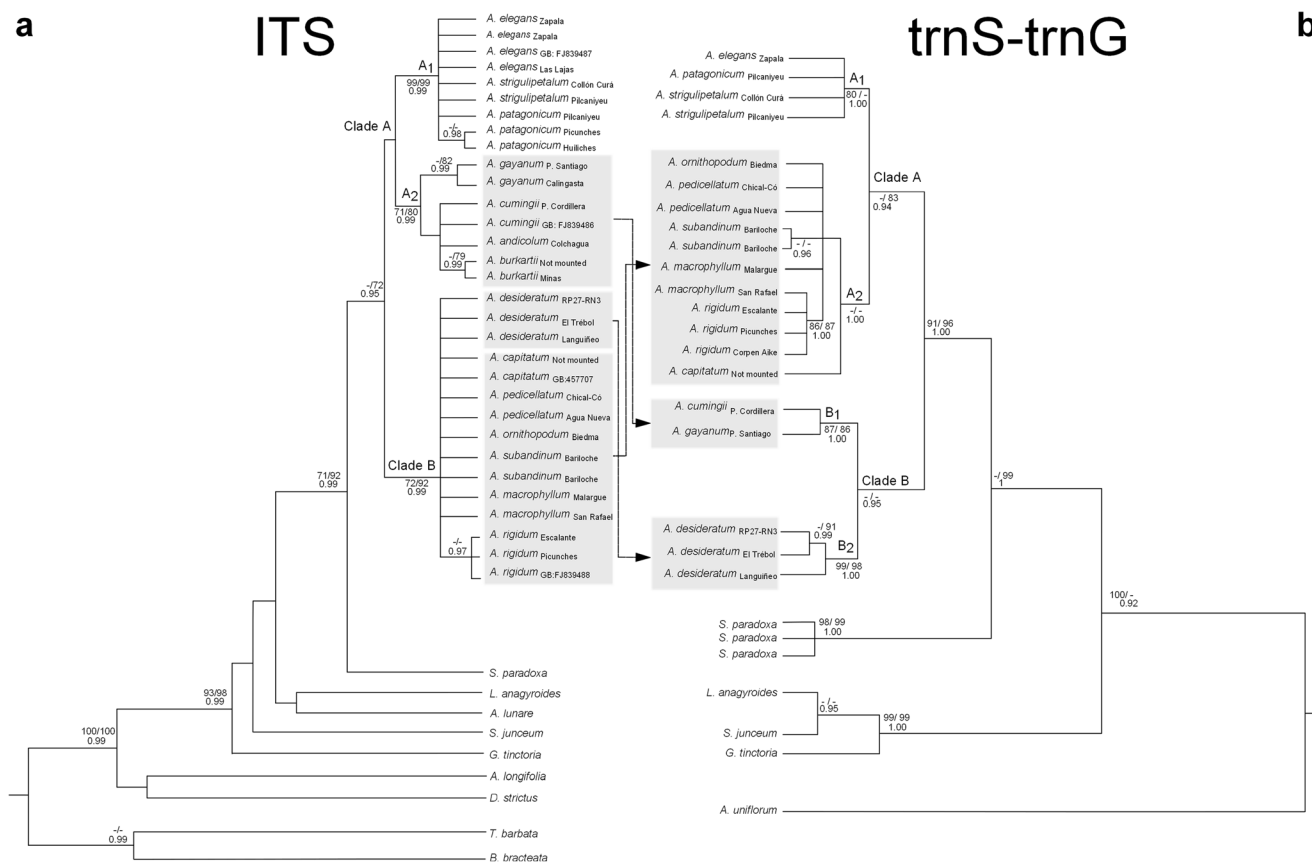


Fig. 3 Comparison of MCC trees based on the ITS and the trnS-trnG sequence data. Values on the branches represent statistical support (BP_{MP}/BP_{ML}/PP). At the nodes, major clades are indicated. Gray boxes and arrows indicate the incongruences between both topologies. The

identified clades are indicated with different shapes: triangle for species belonging to the High Andean Clade (HA), circles for the species belonging to Patagonian Clade 1 (PC1), and squares for the species belonging to the Patagonian Clade 2 (PC2; see Section 3)

times between Patagonian Clade 1 and 2 in Payunia and Patagonian steppe (Fig. 5).

Discussion

Phylogenetic relationships: gene and species trees

This is the first phylogenetic study of *Anarthrophyllum* containing most species included in the genus (14 out of 15 species). We performed an updated phylogenetic approach (gene and species trees) with nuclear and chloroplast molecular markers, which showed a well-supported monophyly of the genus and a geographically concordant topology. Three well-supported main clades were consistently retrieved in association with morphological traits and biogeographical regions: one High Andean and two Patagonian. In the species tree, the High Andean Clade consisted of four geographically circumscribed species which inhabit the slopes of the Andean and Payunia mountain ranges. These species are also distinct in morphology, consisting of prostrate cushions with small leaves, and short, mainly yellow flowers. The other two

clades are distributed across the Patagonian steppe and extend northwards into the Monte Desert across Payunia region; the Patagonian Clade 1 clustered seven species consisting of relatively tall bushes, with yellow flowers, while the Patagonian Clade 2 clustered three species consisting of globular cushions, with red/orange flowers. The species tree provided a stronger and well-structured phylogenetic hypothesis combining the primary topologies of the gene trees and the phenotypic variation in the genus (see below). However, within these three main clades, a low among species phylogenetic resolution was obtained. This low interspecific divergence may reflect relatively rapid speciation within each clade (e.g., Snak et al. 2016).

Although the gene trees retrieved the same main clades observed in the species tree, there were differences between them in relation to the branching order and to the position of *A. desideratum* (see Fig. 3). Several factors may be responsible for the incongruence detected between gene trees; differences in the heritability of the DNA, as nDNA is influenced by pollen flow and cpDNA is under maternal heritability thus influenced by the dispersion of the seeds (Curat et al. 2008). These differences in gene flow may translate into the

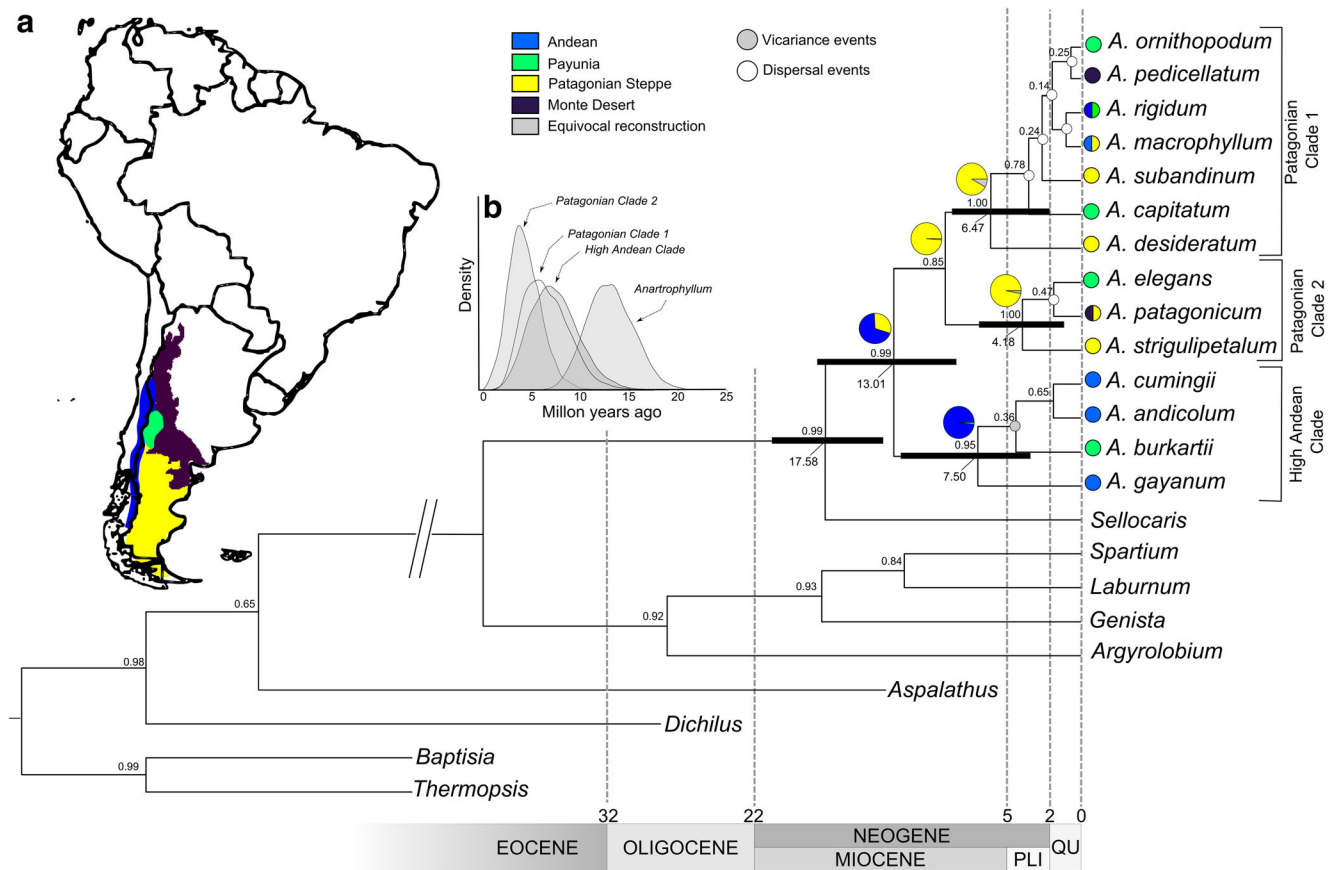


Fig. 4 **a** Calibrated species tree of *Anarthrophyllum*. Values on the branches represent statistical support (PP). Numbers given for each node are the estimated (median) divergence time in million years; black bars indicate 95% highest posterior densities over the median value. Current distributions are indicated by the circles before the species names, and pie graphs on nodes report probabilities after BBM analysis for reconstructions. The map shows the biogeographic regions. Vertical bars indicate major clades explained in the text. **b** Posterior density plots of divergence times for the estimated origin of *Anarthrophyllum* and main clades. QU: Quaternary. PLI: Pliocene

and pie graphs on nodes report probabilities after BBM analysis for reconstructions. The map shows the biogeographic regions. Vertical bars indicate major clades explained in the text. **b** Posterior density plots of divergence times for the estimated origin of *Anarthrophyllum* and main clades. QU: Quaternary. PLI: Pliocene

tree topologies; additionally, both kinds of DNA have differences in their mutational rates and are subjected to several biological intracellular processes such as reticulation, polyploidy, or formation of pseudogenes, which may promote mutations with no phylogenetic information (genetic swamping; Amico 2007; Barker et al. 2005; Currat et al. 2008). The discrepancy in the position of *A. desideratum* samples was not expected; however, in addition to the detected differences retrieved by the molecular markers, the ecology and geographic distribution of this species may contribute to this discrepancy. Future studies including a larger sampling and additional molecular markers will allow to resolve not only the position of *A. desideratum* but also the most probably branching order of the main observed clades.

Biogeography and diversification

Based on the here estimated chronology, divergence between *Anarthrophyllum* and *Sellocaris* occurred 17.58 Ma (21.9–13.7). Although the Andean uplift began about 30 Ma, one of the strongest uplift pulses occurred at the Middle Miocene

(17–15 Ma). By the time *Anarthrophyllum* had diverged, the Andean mountain range had already established a topographic barrier to atmospheric circulation initiating one of the most drastic rain shadows on earth and promoting the relentless aridization of the Patagonian steppe (Houston and Hartley 2003; Blisniuk et al. 2005; Hartley et al. 2005; Hoom et al. 2010). During this period, the diversity and abundance of xerophytic taxa markedly increased in this region, replacing the woodland physiognomy that dominated the landscape during the Paleocene-Oligocene with the present arid bushlands and steppes (Barreda and Palazzesi 2007). Under this scenario of increasing aridity, around 13.01 Ma (15.7–8.7), the major diversification of *Anarthrophyllum* commenced. Species of the genus typical exhibit xeromorphic features such growth form consisting of low (less than 2 m tall) branched bushy or loose to dense cushions and stiff acicular leaves (Soraru 1974). In this line, character reconstruction suggests that the hypothetical ancestors of the genus were shrubs with medium to large leaves probably inhabiting an area between the Andean region (PP = 69%) or Patagonian steppe (PP = 31%), likely associated with high Andean elevations in the

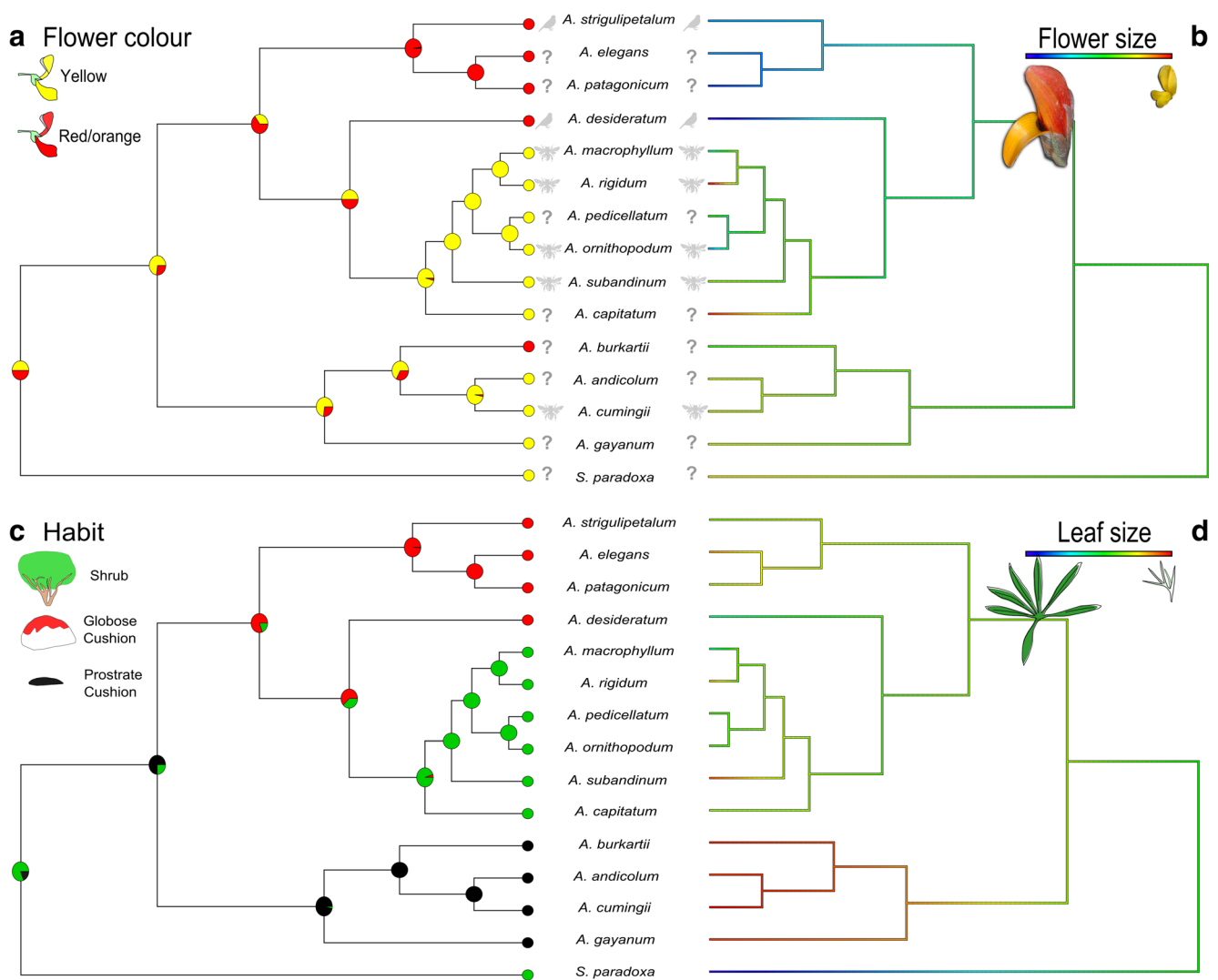


Fig. 5 Stochastic character mapping of categorical characters and continuous trait reconstructions. Current character states are indicated before the species names and pie graphs report posterior probability of each character state on the corresponding node. **a** Flower color. **b** Flower

size. **c** Habit. **d** Leaf length. In **a**, floral color was coded as 0 (yellow) and 1 (red/orange) and in **c** habit as 0 (shrub), 1 (globular cushion), and 2 (prostrate cushion)

late Tertiary and the associated hyperaridization of the continent. These ancestors likely gave rise to two main clades, one which got established in the high Andean region diversifying into the High Andean Clade and the other in the wide Patagonian steppe giving rise to the Patagonian Clades.

The High Andean Clade diversified around 7.5 Ma (12.7–3.0) in mountain landscapes; this clade of four species is well-supported as monophyletic: *A. andicolum*, *A. gayanum*, and *A. cumingii* are distributed in the High Andean region between 2400 and 3400 m altitude, while the fourth species of this clade, *A. burkartii*, moved to the lower Payunia regions (\approx 2000 m) that are climatically similar to the High Andean region (Martínez Carretero 2004). This biogeographical pattern is consistent with the proposal of the Andean mountain range as a dispersal center to extra-Andean mountain systems, such

as Payunia (Roig-Juñent et al. 2007, 2008). Allopatric speciation likely occurred subsequently though limited gene flow, mutations, and genetic drift as populations became isolated geographically (e.g., in arid land of South America: Cosacov et al. 2009; Särkinen et al. 2012; Chacón et al. 2012). Strongest geographic isolation would have taken place during the last of the Andean uplifts 11–5.3 Ma (Ortiz-Jaureguizar and Cladera 2006; Ramos et al. 2014), with the Andes forming a geographic barrier to the ancestral populations of *Anarthrophyllum*. Consequently, species geographical distribution and altitudinal range affected diversification of the High Andean Clade promoting allopatric speciation. *Anarthrophyllum* is particularly prone to diversification by vicariance since pods open explosively, dispersing the seeds few meters away from the plants (see Sorarú 1974).

The High Andean Clade species share several phenotypic features which suggest their adaptation to the microclimatic conditions at high altitudes and which represent evolutionary innovations with respect to the ancestral state of the genus. Such features are the low prostrate cushion-like growth form and the small leaves (less than 8.5 mm) reportedly adapted to environmental stressful conditions as low temperatures, strong winds, and drought which prevail in high mountain regions (Ruthsatz 1978; Cavieres and Peñaloza 1998). Regarding the floral phenotype, this clade retrieved species with short and yellow bee-pollinated flowers, similar to the reconstructed ancestor of *Anarthrophyllum*. This suggests that the High Andean Clade conserve the ancestral pollination syndrome. Bee-pollinated flowers are also reported as the ancestral state in other genera of Legume clades (e.g., in *Gastrolobium*; Chandler et al. 2001).

The two Patagonian ancestors bearing a globular cushion habit with medium to large leaves and relatively large red/orange flowers. The Patagonian Clade 1 includes seven species (*A. desideratum*, *A. ornithopodum*, *A. pedicellatum*, *A. rigidum*, *A. macrophyllum*, *A. subandinum*, and *A. capitatum*) with *A. desideratum* sister to the other species of the clade. This clade originated and diversified during the expansion of the Patagonian steppe around 6.47 Ma (11.2–3.4) when the Andean range reached its final altitude and the already progressing aridization further increased. This favored the establishment of the current steppe dominated by grasses and shrubs (Ortiz-Jaureguizar and Cladera 2006; Barreda et al. 2008). These results agree with previous studies that associated intraspecific lineage diversification with the origin of the Patagonian steppe (Jakob et al. 2009; Cosacov et al. 2010, Cosacov et al. 2013; Sede et al. 2012).

One species of the Patagonian Clade 1 (*A. pedicellatum*) moved to the Monte Desert, while two (*A. ornithopodum* and *A. capitatum*) reached Payunia region and contacted *A. burkartii* of a different clade. As S-DIVA analysis indicated, the greatest number of dispersal events was observed within this clade. Possibly, these dispersions took place in the Patagonian steppe (Barreda et al. 2008) and southernmost Monte Desert regions (Labraga and Villalba 2009) about 5 Ma ago when the expansion of arid environments enabled dispersion of taxa to new habitats facilitating differentiation through isolation by distance. Most species of this clade are phenotypically characterized as up to 2-m-tall shrubs, varying in leaf size, with medium to short yellow flowers. The shrub habit which appeared exclusively in this clade was presumably favorable for the colonization of the most arid portions of the northern Patagonian steppe and the warmer southern Monte Desert (Oliva et al. 2001). A significant change in habit was observed in *A. desideratum*, the most divergent species of the clade, since it is the only one presenting a globular cushion habit and large red/orange flowers. This plant architecture appears to be wind resistant (Ezcurra et al. 1991), which is

consistent with the distribution of this species of windy open areas of the Patagonian steppe at the southernmost part of the range of the genus. This species has been reported as bird-pollinated (Paiaro 2010) and exhibits an evident ornithophilous flower syndrome. The remaining species of the clade are likely pollinated by bees, as has been recorded for *A. macrophyllum*, *A. ornithopodum*, *A. rigidum*, and *A. subandinum* (A. Sérsic, personal observation). The differentiation of *A. desideratum* within this clade is clearly associated with the transition from bee to bird pollination. Transitions in this direction have been frequently recorded elsewhere in environments with inclement weather unfavorable for insect pollinators (Cruden 1972; Cronk and Ojeda 2008; Thomson and Wilson 2008).

The Patagonian Clade 2 retrieved three species (*A. strigulipetalum*, *A. patagonicum*, and *A. elegans*) that diversified in the Patagonian steppe more recently, around 4.18 Ma (5–0.6) at the beginning of the Pliocene and during the Quaternary. Plant communities were affected during these periods by climatic fluctuations associated to glacial and interglacial cycles (Gibbard and Cohen 2008). In this line, the species of this clade are presently distributed in areas postulated as Quaternary periglacial refugia, where lineages may have been isolated and become speciated (see Cosacov et al. 2010, 2013). Character reconstructions revealed a common ancestor consisting of globular cushions with medium-sized leaves and large red-orange flowers. Although bird pollination has been recorded only for *A. strigulipetalum* (Sérsic, personal observation), the remaining species of the clade also have flower features consistent with bird pollination, suggesting that a pollinator shift could have also triggered diversification in this clade.

Stochastic character mapping suggests that large red-orange flowers and the globular cushion habit evolved independently in *A. desideratum* and within the Patagonian Clade 2. These lineages share a similar floral phenotype regarding flower size and color. Some evolutionary transitions between pollination syndromes are more frequently reported than others, with the transition from bee to bird pollination prevailing (revisited in van der Niet and Johnson 2012). This transition involves changes in flower morphology, for example, changes to narrower, longer, and red corollas such as those observed within these two clades of the genus. The present finding represents additional independent bee-to-bird pollinator transitions of plant clades that colonized the Patagonian steppe. A similar transition has been reported before for Calceolariaceae where *Calceolaria uniflora* Lam. represents a finch pollinated descendant from an Andean clade where pollination by oil-collecting bees dominates (Cosacov et al. 2009). Likewise, such transition in *Calceolaria* has involved shifts to a bird-adapted flower phenotype. Presumably in these plant clades, pollination by birds was more reliable than bee pollination when colonizing the insect impoverished steppe.

Conclusions

Despite the inconsistencies between cpDNA and nDNA trees, phylogenetic results retrieved consistently three main lineages within the genus *Anarthrophyllum*, one High Andean and two of the Patagonian steppe. Main diversifications occurred concomitant with the Andean uplift and its ecological consequences: the Andean mountain range appears at one hand, as a species barrier for the High Andean clade, and on the other hand, it produced a rain-shadow effect that resulted in the establishment of the extremely xeric regions of South America, such as the Monte and the Patagonian steppe. Concomitant with these effects, vegetative traits showed adaptations to harsh climates in some clades, while floral features, more related to their pollinators, would have influenced the diversification of other lineages. Thus, clades' delimitation (leaving aside the unstable placement of *A. desideratum*) has been supported alternatively by morphological, ecological, historical, and geographical factors forming a complex evolutionary and biogeographic history for the genus.

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