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Reconstructing the phylogenetic history of the tribe Leucocoryneae (Allioideae): Reticulate evolution and diversification in South America

Agostina B. Sassone*, Liliana M. Giussani

Instituto de Botánica Darwinion, CONICET-ANCEFN, Labardén 200, CC 22, B1642HYD, San Isidro, Buenos Aires, Argentina

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ABSTRACT

At present, the Allioideae is included within the Amaryllidaceae, which is an economically important bulb crop subfamily that includes onion, garlic, and ornamental species worldwide. The Allioideae includes four tribes geographically disjunct namely: Allieae, widespread in the northern hemisphere, tribe Tulbaghieae distributed in South Africa, and tribes Leucocoryneae and Gilliesieae are endemic to South America. Although we agree with the current tribal circumscription of the Leucocoryneae including *Beauverdia*, *Ipheion*, *Latace*, *Leucocoryne*, *Nothoscordum,* and *Tristagma*, there are still taxonomic and phylogenetic uncertainties regarding the monophyly, phylogenetic relationships, and divergence time of several lineages in a biogeographic context. In this study, a comprehensive molecular phylogeny of the tribe Leucocoryneae was inferred based on nuclear ribosomal ITS and plastid (*ndhF* and *matK*) sequences. We used Bayesian inference and maximum parsimony analyses to predict ancestor-descendant relationships. Our results confirmed the monophyly of the four tribes of subfamily Allioideae. Similarly, within the Leucocoryneae, *Ipheion*, *Leucocoryne,* and *Nothosocordum* Sect. *Inodorum* were also monophyletic; *Tristagma* and *Nothoscordum* would be monophyletic if including *Ipheion* and *Beauverdia*, respectively. Network analyses were implemented to reveal putative scenarios of reticulate evolution. Both, current and ancestral hybridization events have presumably occurred among species of *Nothoscordum* Sect. *Nothoscordum* and *Beauverdia* favored by spatial overlapping of populations, flowering synchrony and a puzzling pattern of cytogenetic attributes. The estimation of divergence time indicates that the tribe Leucocoryneae originated in the Late Oligocene in southern South America with possible ancestors in Africa. Most crown lineages within the tribe diversified in conjunction with biogeographical events during the Late Miocene to Pliocene. We posit that new suitable environments available after the Andean uplift and during the Age of the Southern Plains provided the favorable geographic setting for the major lineages of Leucocoryneae in southern Pampas, extra-Andean Patagonia, Andean mountains, and in Chile. Hybridization, polyploidization, and Robertsonian translocations of chromosomes have been the driving forces and major sources of speciation in the evolution of tribe Leucocoryneae.

1. Introduction

Phylogenetic relationships within Amaryllidaceae have been investigated for the last two decades (e.g., Fay and Chase, 1996; Meerow et al., 2000; Chase and Reveal, 2009). As a result, the circumscription of different taxa has changed leading to the recircumscription of subfamilies, tribes, subtribes, genera, and species. The current classification schemes outline the Amaryllidaceae as a family of approximately 90 genera and 1800 species (*fide* Jin, 2013), divided into three subfamilies: Amarylloideae, Agapanthoideae, and Allioideae. Within the Allioideae, four monophyletic tribes have been recognized: Allieae (Nguyen et al., 2008), Tulbaghieae (Stafford and Rønsted, 2015), Gilieasieae (Escobar, 2012), and Leucocoryneae (Sassone et al., 2014a). The tribe Allieae is widespread in the northern hemisphere; tribe Tulbaghieae is distributed in South Africa, while the tribes Leucocoryneae and Gilliesieae are endemic to South America. *Nothoscordum bivalve* (L.) Britton is the only species reaching Mexico and USA. Most treatments recognize the following phylogenetic relationships: [Allieae [Tulbaghiea, [Gilliesieae, Leucocoryneae]]]. Recently, Pellicer et al. (2017) proposed Gilliesiinae and Leucocoryninae to be subtribes of Gilliesieae. The circumscription of subtribe Leucocoryninae is however, confusing. Fay and Christen (in Pellicer et al., 2017), when defining the subtribe, referred to the original circumscription of Ravenna (2001a), who originally included two divergent genera: *Leucocoryne* Lindl. and *Tulbaghia* L. by having floral appendices. Therefore, in this study we followed Sassone et al. (2014a), who amended the tribal circumscription of

* Corresponding author.
E-mail address: asassone@darwin.edu.ar (A.B. Sassone).

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Leucocoryneae to include 6 genera.

The tribe Leucocoryneae is distinguished from its sister group Gilliesieae by two synapomorphies: the presence of actinomorphic flowers and septal nectaries (Rudall et al., 2002; Sassone et al., 2014a). This tribe consists of six South American genera with ca. 100 species (Sassone et al., 2014a): *Beauverdia* Herter (4 spp, Sassone et al., 2014b), *Ipheion* Raf. (3 spp., Sassone et al., 2014a), *Latace* Phil. (2 spp, Sassone et al., 2015), *Leucocoryne* (15 spp, Muñoz and Moreira, 2000), *Nothoscordum* Kunth (20–80 spp.), and *Tristagma* Poepp. (12 spp; Arroyo-Leuenberger and Sassone, 2016). In addition, a wide variation in morphological and karyological features is characteristic of the Leucocoryneae (Guaglianone, 1972; Sassone et al., 2013, Pellicer et al., 2017; Sassone et al., 2018). In terms of phenological attributes, species of Leucocoryneae present early flowering times, in general, they are part of the earliest flowering plants in the regions they are occurring in (Guaglianone, 1972; Sassone, 2017). Some species of *Nothoscordum* have been reported to present two flowering periods per year: one in autumn and the other in spring (Guaglianone, 1972). Furthermore, the flowering month has been used as a diagnostic character to discriminate similar species within *Nothoscordum*, namely *N. gaudichaudianum* Kunth *vs. N. bivalve* (L.) Britton (Guaglianone, 1972).

Recently, the monophyly of the tribe Leucocoryneae has been corroborated by molecular data (Souza et al., 2016; Pellicer et al., 2017). The monophyly of genera within the tribe has been confirmed or rejected based on different studies (Jara-Arancio et al., 2014; Souza et al., 2015, 2016; Pellicer et al., 2017), and the phylogenetic reconstruction of cytological and morphological characters have helped to better understand relationships among lineages. For instance, *Leucocoryne* has been proposed as a monophyletic group including *Pabellonia* Quezada & Martic. and *Stemmatium* Phil. (Jara-Arancio et al., 2014; Souza et al., 2015). Likewise, *Latace* (=*Zoellnerallium* Crosa, Sassone et al., 2015) has been validated as the sister group of *Leucocoryne*, which has also been inferred as a monophyletic entity (Souza et al., 2016). Based on molecular data, *Nothoscordum* is paraphyletic because includes *Beauverdia* (Souza et al., 2016; Pellicer et al., 2017). However, based on morphological characters, the monophyly of *Beauverdia* is strongly supported (Sassone et al., 2014b). In turn, *Tristagma* and *Ipheion* (as circumscribed by Arroyo-Leuenberger and Sassone 2016, Sassone and Arroyo-Leuenberger ined.) were resolved as monophyletic sister groups (Souza et al., 2016; Pellicer et al., 2017). However, previous studies have included few representatives of both genera. Furthermore, high cytogenetic variation has been reported among genera and species of the Leucocoryneae (see Sassone et al., 2018 and references therein). That is, cytological variation comprises changes in base chromosome numbers ranging from $x = 4$ to $x = 12$, ploidy levels varying from 2*x*, 3*x*, 4*x*, and 6*x* chromosome sets, fundamental numbers varying from 14 to 48, different karyotype formula varies mainly as a result of chromosomal rearrangements (fission-fusion events), DNA content, and 1C*x* fluctuating from 9 to 30 pg (Pellicer et al., 2017; Sassone et al., 2018).

In plants, incomplete lineage sorting, horizontal gene transfer, and hybridization are the putative main causes associated with non-tree-like phylogenetic patterns (Linder and Rieseberg, 2004; McBreen and Lockhart, 2006). Reticulate evolutionary relationships are expected to be prevalent among closely related species that are not completely reproductively isolated (Eaton and Ree, 2013). Moreover, in the Leucocoryneae, natural hybridization events and speciation resulting from allopolyploidization have been reported (Crosa, 1974; Nuñez, 1990; Souza et al., 2016; Pellicer et al., 2017).

Because no fossil record is known for the Amaryllidaceae (Smith, 2013), few attempts have been made to infer the evolutionary time of speciation within subfamily Allioideae, in particular lineages of the tribe Allieae. Based on an ITS phylogeny, Dubouzet and Shinoda (1999) estimated that the divergence among Old and New World *Allium* would have occurred between 50 and 25 Ma. However, a recent study estimated the origin of *Allium* during the Late Eocene, ca. 34.26 Ma (Li et al., 2016). Similarly, in an attempt to estimate the divergence time of biome shifts in *Leucocoryne*, Jara-Arancio et al. (2014) set the origin of Leucocoryneae at a similar age around 37–25 Ma.

In summary, even though several studies have included different taxonomic sampling, there are still phylogenetic conflicts to be clarified within the tribe Leucocoryneae. The aims of the present study were: (1) to test the monophyly of genera and species of tribe Leucocoryneae including an exhaustive taxonomic sampling, (2) to elucidate the generic and specific phylogenetic relationships, (3) to estimate the divergence time in the evolution of the tribe, (4) to explore putative reticulation events, and (5) to interpret phylogenetic relationships based on previous morphological, phenological and/or cytological data. In order to answer these inquiries, we generated molecular phylogenies based on ITS and plastid (*ndhF* and *matK*) sequences involving a comprehensive sampling of the tribe Leucocoryneae and representative taxa of all other tribes in subfamily Allioideae.

2. Materials and methods

2.1. Plant materials

Field trips were carried out in central and southern Chile, southern and eastern Uruguay, and the Argentinian provinces of Buenos Aires, Entre Ríos, Mendoza, Neuquén, Río Negro, and Santa Cruz. The taxonomic sampling includes representatives of all genera of the tribe Leucocoryneae, namely all species of *Beauverdia*, all species of *Ipheion,* 8 species of *Nothoscordum* representing the two sections: Sect. *Nothosocordum*, and Sect. *Inodorum*, 7 species of *Tristagma,* 5 representative species of *Leucocoryne,* and one species of *Latace* (Table 1).

2.2. DNA Isolation, Amplification, and Sequencing

Genomic DNA was isolated from silica-dried leaf tissue following a modified CTAB protocol (Doyle and Doyle, 1987) or from herbarium material with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Based on the resolution obtained by previous results for genetic markers in Asparagales (Ito et al., 1999; Seberg et al., 2012), the plastid genes, *matK* (encoding the maturase K protein) and *ndh*F (NADH dehydrogenase F) were selected. As a nuclear DNA region, the ribosomal internal transcribed spacer (ITS) was also studied. The latter marker has been broadly used in phylogenetic analyses in plants, and it has been reported as one of the most informative nuclear markers (Kwembeya et al., 2007; Choi et al., 2012). The plastid gene *matK* was amplified using AF and 8R primers (Ito et al., 1999). The *ndh*F gene was amplified in three fragments using the following primer sets: 5F-972R, 972-1666R, 1318F-3R. The ITS region was amplified using ITS2, ITS3, ITS4 and ITS5 (Baldwin et al., 1995).

DNA amplification was conducted in a volume of 25 µl polymerase chain reaction (PCR) containing 20–40 ng of DNA template and a final concentration of 1xPCR Buffer minus Mg, 2.5 mM $MgCl_2$, 0.025 mM of each dNTP, 0.2 µM of both forward and reverse primers and 1.25–2.5 units of Taq Polymerase (Invitrogen Life Technologies, São Paulo, Brazil). PCR amplifications were performed under the following conditions: (1) ITS:1 cycle of 94 °C for 5 min, 34 cycles of 94 °C for 30 s, 53 °C for 1 min, and 72 °C for 1.5 min, and a final extension cycle of 72 °C for 7 min, (2) *matK* was amplified according to Ito et al. (1999): 1 cycle of 94 °C for 5 min, 34 cycles of 94 °C for 30 s, 53 °C for 1 min, and 72 °C for 1.5 min and extension at 72 °C for 2 min. (3) For *ndh*F we used 1 cycle of 94 °C for 4 min, 34 cycles of 94 °C for 1 min, 48 °C for 1 min, and 72 °C for 2.5 min, and a final extension step of 72 °C for 7 min. When species for which amplification protocols were not successful a variety of PCR additives and enhancing agents (bovine serum albumin, dimethyl sulfoxide, formamide) were used to increase the yield, specificity and consistency of PCR reactions. Sequencing reactions were performed by Macrogen, Inc. using the ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (Applied Biosystems, Seoul, Korea), following the protocols supplied by the

Table 1
List of taxa investigated in this study including voucher information, citation information, and Genbank accession numbers.

Taxon	Voucher	Collection Place	ITS	ndhF	matK
Allium cepa	BF-ALL-047	Slovenia	FJ664287	–	–
Allium sativum	BF-ALL-037	–	EU626375	–	–
Allium triquetrum	Morrone, O. 6252	Argentina. Pcia. Bs. As. Tandil	MH159815	–	–
Beauverdia dialystemon	Giussani, L. 501	Argentina. Pcia. Buenos Aires. Gonnet	MH159822	MH159893	–
Beauverdia dialystemon	Castillo, A. s.n.	Cultivada. Procedencia desconocida	MH159821	MH159892	MH159944
Beauverdia dialystemon	Giussani, L. 500	Argentina. Pcia. Buenos Aires. La Plata. Cdad. De los Niños	MH159823	–	–
Beauverdia dialystemon	RBGK 1989-2632	Pellicer et al. (2017)	LT718268	–	–
Beauverdia dialystemon	RBGK 1984-3093	Pellicer et al. (2017)	LT718329	–	–
Beauverdia hirtella	RBGK 1991-1506	Pellicer et al. (2017)	LT718333	LT718395	LT718271
Beauverdia hirtella	RBGK 1987-3395	Pellicer et al. (2017)	LT718332	LT718394	LT718270
Beauverdia hirtella	RBGK 1986-3905	Pellicer et al. (2017)	LT718331	LT718393	LT718269
Beauverdia hirtella subsp. hirtella	Giussani, L. 482	Uruguay. Lavalleja. Cerro Arequita	MH159825	MH159891	MH159943
Beauverdia hirtella subsp. hirtella	Giussani, L. 468	Uruguay. Lavalleja. Cerro Arequita	MH159826	–	–
Beauverdia hirtella subsp. lorentzii	Giussani, L. 424	Argentina. Pcia. Entre Ríos. Complejo Termas de Concepción.	MH159824	MH159890	MH159942
Beauverdia sellowiana	Castillo, A. s.n.	Cultivada. Procedencia: Uruguay.	MH159827	MH159894	MH159945
Beauverdia sellowiana	Giussani, L. 462	Uruguay. Minas. Cerro Verdún.	MH159828	MH159895	MH159946
Beauverdia sellowiana	Giussani, L. 465	Uruguay. Minas. Cerro Verdún. (8 tepalos)	MH159829	MH159896	MH159947
Beauverdia vittata	Giussani, L. 429	Argentina. Pcia. Entre Ríos. Uruguay. Colonia Elia	MH159830	MH159897	MH159948
Beauverdia vittata	Giussani, L. 431	Argentina. Pcia. Entre Ríos. Uruguay. Camino de Colonia Elia a Puerto Campichue	MH159831	MH159898	MH159949
Beauverdia vittata	Giussani, L. 481	Uruguay. Lavalleja. Cerro Arequita	MH159832	MH159899	MH159950
Beauverdia vittata	RBGK 2006-1196	Pellicer et al. (2017)	LT718345	LT718407	LT718283
Gilliesia graminea	Chase,M. 450	Chile	HQ393006	–	–
Gilliesia graminea	RBGK 1977-2342	Pellicer et al. (2017)	LT718327	LT718389	LT718265
Ipheion sessile	Castillo, A. s.n.	Cultivada. Uruguay.	MH159851	MH159900	MH159951
Ipheion sessile	Giussani, L. 469	Uruguay. Minas. Cerro Verdún.	MH159850	MH159901	MH159952
Ipheion sessile	Giussani, L. 487 a	Uruguay. San José.	MH159852	–	–
Ipheion sessile	Giussani, L. 487 b	Uruguay. San José.	MH159853	–	–
Ipheion tweedieanum	Giussani, L. 420	Argentina. Pcia. Entre Ríos. Ruta Nac. 14. Arroyo Gualeyán	MH159854	MH159902	MH159953
Ipheion tweedieanum	Giussani, L. 488	Uruguay. San José. Ruta 24, de Fray Bentos a Paysandú.	MH159855	MH159903	–
Ipheion uniflorum	Múlgura, M. E. 4587	Argentina. Pcia. Buenos Aires: Saladillo	MH159857	MH159904	MH159954
Ipheion uniflorum	Morrone, O. 6339	Argentina. Pcia. Buenos Aires: Tandil	MH159858	MH159905	MH159955
Ipheion uniflorum	RBGK 2004-3197	Pellicer et al. (2017)	LT718338	LT718400	LT718276
Ipheion uniflorum	Giussani, L. 496	Argentina. Pcia. Buenos Aires: San Isidro.	MH159856	–	–
Ipheion uniflorum	RBGK 2000-2528	Pellicer et al. (2017)	LT718341	LT718403	LT718279
Ipheion uniflorum	RBGK 1988-3378	Pellicer et al. (2017)	LT718340	LT718402	LT718278
Ipheion uniflorum	RBGK 1978-2423	Pellicer et al. (2017)	LT718335	–	–
Ipheion uniflorum	RBGK 1994-160	Pellicer et al. (2017)	LT718336	–	–
Latace andina	Zuloaga, F. 12378	Argentina. Pcia. Mendoza. Hotel Termas del Sosneado	MH159820	MH159889	MH159941
Latace andina	Zuloaga, F. 15126	Argentina. Pcia. Neuquén. Minas.Ruta Prov. 54	MH159819	–	–
Latace andina	Jara-Arancio 38	Chile	KF171082	–	–
Latace andina	Giussani, L. 625	Chile	MH159818	–	–
Leucocoryne coquimbensis	RBGK 2006-38	Chile	LT718353	LT718415	LT718291
Leucocoryne ixioides	Johnson, L. 10-131	Chile. Santiago. Camino a Farellones	MH159817	MH159888	MH159940
Leucocoryne narcissioides	RBGK 1992-1051	Pellicer et al. (2017)	LT718363	LT718425	LT718301
Leucocoryne pauciflora	RBGK 1977-6123	Pellicer et al. (2017)	LT718366	LT718428	LT718304
Leucocoryne purpurea	RBGK 1987-4154	Pellicer et al. (2017)	LT718369	LT718431	LT718307
Lycoris radiata	Unkown	Unkown	JX975647	–	–
Lycoris sprengeri	Unkown	Unkown	AY942716	–	–
Miersia chilensis	RBGK 2003-3578	Pellicer et al. (2017)	LT718373	–	–
Nothoscordum andicolum	Zuloaga, F. 13116	Argentina. Jujuy. Santa Catalina.	MH159833	MH159928	MH159975
Nothoscordum andicolum	RBGK 1986-104	Pellicer et al. (2017)	LT718374	LT718435	LT718312
Nothoscordum arenarium	Morrone, O. 6301	Uruguay. Colonia. Pque. Forestal Ferrando.	MH159834	MH159929	MH159976
Nothoscordum bivalve	Noriega 16	México. Durango. El Carmen	MH159835	MH159930	MH159977
Nothoscordum bonariense	Morrone, O. 6247	Argentina. Pcia. Buenos Aires: Tandil. Cerro de las Animas.	MH159836	MH159931	MH159978
Nothoscordum bonariense	Giussani, L. 450	Argentina. Pcia. Buenos Aires: Magdalena. Arroyo Juan Blanco	MH159837	MH159932	MH159979
Nothoscordum bonariense	Giussani, L. 504	Uruguay. San José. Ruta 24, de Fray Bentos a Paysandú.	MH159838	MH159933	MH159980
Nothoscordum bonariense	Giussani, L. 426	Ruta Nac. 14. Complejo Termas Concepción, frente a la cabaña 4	MH159841	MH159936	MH159983
Nothoscordum gracile	Morrone, O. 6221	Argentina. Pcia. Buenos Aires: General Pueyrredón. Sierra de los Padres	MH159839	MH159934	MH159981
Nothoscordum montevidense	Villamil, C. 11687	Argentina. Pcia. Buenos Aires: Bahía Blanca	MH159843	–	–
Nothoscordum montevidense	Morrone, O. 6317	Uruguay. Lavalleja. Minas. Cerro Verdún.	MH159840	MH159935	MH159982
Nothoscordum montevidense	RBGK 1985-2643	Pellicer et al. (2017)	LT718375	LT718436	LT718313
N montevidense var minarum	RBGK 1976-3834	Pellicer et al. (2017)	LT718376	LT718437	LT718314
N montevidense var minarum	Morrone, O. s.n.	Uruguay. Maldonado	MH159842	MH159937	MH159984
Nothoscordum nudicaule	Giussani, L. 452	Argentina. Pcia. Buenos Aires	MH159845	MH159938	MH159985
Nothoscordum nudicaule	Peralta s.n.	Argentina. Pcia. Mendoza. Luján de Cuyo	MH159846	–	–
Nothoscordum nudicaule	Giussani, L. 506	Argentina. Pcia. Buenos Aires: Gonnet	MH159847	–	–
Nothoscordum sp.1	Deginani, N. 2180	Argentina. Pcia. Mendoza. Tunuyán. Manzano Histórico.	MH159849	MH159927	MH159974
Nothoscordum sp. 2	Giorgetti, M.	Argentina. Pcia. Salta. Angastaco	MH159848	–	–
Nothoscordum sp. 3	Sassone, A. 24	Argentina. Pcia. Mendoza. Luján de Cuyo	MH159844	–	–
Nothoscordum sp. 4	RBGK 2003-2563	Pellicer et al. (2017)	LT718377	LT718438	LT718315
Solaria atropurpurea	RBGK 1988-1970	Pellicer et al. (2017)	LT718324	–	–
Solaria atropurpurea	Chase, M. 693	Chile	HQ393007	–	–

(*continued on next page*)

Table 1 (*continued*)

Taxon	Voucher	Collection Place	ITS	ndhF	matK
Solaria miersioides	Zuloaga, F. 12510	Argentina. Pcia. Neuquén. Minas. Lagunas de Epu-Lauquen	MH159816	MH159887	MH159939
Solaria miersioides	RBGK 2008-3049	Pellicer et al. (2017)	LT718378	LT718439	LT718316
Tristagma ameghinoi	Zuloaga, F. 15321	Argentina. Pcia. Mendoza. Luján de Cuyo. Vallecitos	MH159875	MH159907	MH159956
Tristagma bivalve	RBGK 1979-783	Pellicer et al. (2017)	LT718380	LT718440	LT718318
Tristagma bivalve	RBGK 1988-8211	Pellicer et al. (2017)	LT718382	LT718442	LT718320
Tristagma bivalve	RBGK 1988-1689	Pellicer et al. (2017)	LT718383	LT718443	LT718321
Tristagma bivalve	Giussani, L. 624	Chile. Santiago. Desde Santiago a Farellones. Curva n° 33.	MH159876	MH159919	MH159966
Tristagma bivalve	Giussani, L. 647	Chile. Biobío. Ñuble. Valle Las Trancas	MH159881	MH159920	MH159967
Tristagma bivalve	Giussani, L. 631	Chile. Maipo. Ruta G 25. Cajón del Maipo.	MH159879	MH159921	MH159968
Tristagma bivalve	Giussani, L. 645	Chile. Biobío. Ñuble. Valle Las Trancas	MH159880	MH159922	MH159969
Tristagma bivalve	Pfanzelt 166		MH159925	MH159972	–
Tristagma bivalve	Giussani, L. 629	Chile. Santiago. Desde Santiago a Farellones. Curva n° 32	MH159884	–	–
Tristagma bivalve	Giussani, L. 646	Chile. Biobío. Ñuble. Valle Las Trancas	MH159882	–	–
Tristagma bivalve	Moreira 1087 (SGO)	Chile. Maule. Vilches, Reserva Nacional Lircay	MH159873	MH159918	
Tristagma circinatum	Zuloaga, F. 12356	Argentina. Pcia. Mendoza. Las Leñas	MH159861	MH159908	–
Tristagma circinatum	Sassone, A. 34	Argentina. Pcia. Mendoza. Las Leñas a Valle Hermoso.	MH159863	MH159910	MH159958
Tristagma circinatum	Zuloaga, F. 15199	Argentina. Pcia. Mendoza. Las Leñas a Valle Hermoso.	MH159864	–	–
Tristagma gracile	Giussani, L. 650	Chile. Biobío. Camino a Reserva del Ñuble. Valle del Río Digullin.	MH159883	MH159926	MH159973
Tristagma gracile	Muñoz, M. 4122	Chile. Santiago. Bahía Catalina a Pintué.	MH159860	–	–
Tristagma graminifolium	Montero 2440 (CONC 130289)	Chile. Cerro Renca.	MH159865	–	–
Tristagma graminifolium	Giussani, L. 637	Chile. Santiago. Cerro Renca.	MH159878	MH159923	MH159970
Tristagma nivale	Zavala-Gallo, L. 102	Argentina. Pcia. Río Negro. Bariloche. Cima del C° Challhuaco	MH159867	MH159912	MH159960
Tristagma nivale	Zuloaga, F. 15014	Argentina. Pcia. Neuquén. Loncopué. Copahue.	MH159870	MH159913	MH159961
Tristagma nivale	Zuloaga, F. 15138	Argentina. Pcia. Neuquén. Minas	MH159871	MH159914	MH159962
Tristagma nivale	Jara-Arancio 15729	Chile	KF171083	–	–
Tristagma nivale	Humano, s.n. (1)	Argentina. Pcia. Santa Cruz	MH159868	–	–
Tristagma nivale	Covieres 3704 (CONC 160213)	Chile	MH159869		–
Tristagma nivale	Humano, s.n. (2)	Argentina. Pcia. Santa Cruz	MH159886	–	–
Tristagma patagonicum	Zuloaga, F. s.n.	Argentina. Pcia. Mendoza. Sa. del Nevado	MH159859	MH159906	–
Tristagma patagonicum	Rafael, M. 48	Argentina. Pcia. Chubut. Futaleufú. Esquel	MH159862	MH159909	MH159957
Tristagma patagonicum	Sassone, A. 30	Argentina. Pcia. Mendoza. Camino a Hotel y Termas el Sosneado.	MH159866	MH159911	MH159959
Tristagma patagonicum	Rafael, M. 49	Argentina. Pcia. Chubut. Futaleufú. Esquel	MH159874	MH159915	MH159963
Tristagma patagonicum	Zuloaga, F. 12534	Argentina. Pcia .Neuquén. Minas. Desvío de la Ruta Provincial 43	MH159872	MH159916	MH159964
Tristagma patagonicum	Sassone 21	Argentina. Pcia. Neuquén.Zapala. Parque Nacional Laguna Blanca	–	MH159917	MH159965
Tristagma violaceum	Giussani, L. 652	Chile. Biobío. Ñuble. Valle del Río Digullin.	MH159877	MH159924	MH159971
Tulbaghia capensis	H.J.Choi s.n. (KH)	–	GQ412258	–	–
Tulbaghia ludwigiana	RBGK 1988-1970	Pellicer et al. (2017)	LT718384	LT718444	LT718322
Tulbaghia simmleri	Chase 17513	Pellicer et al. (2017)	LT718385	LT718445	–
Tulbaghia violacea	JC Pires 2011	–	–	JQ276781	JQ276393
Tulbaghia violacea	–	India	KT373964	–	–

manufacturer.

A total of 174 sequences were generated in this study. Almost all sequences correspond to species of Leucocoryneae plus three sequences of the outgroup (*Allium triquetrum* L., *Miersia chilensis* Lindl., and *Solaria miersioides* Phil.); the remaining sequences were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and detailed in Table 1. *Lycoris radiata* (L'Hér.) Herb., *L. sprengeri* Comes ex Baker (Amaryllidaceae), *Allium cepa* L., *A. sativum* L. and *A. triquetrum* (tribe Allieae), *Tulbaghia capensis* L., *T. ludwigiana* Harv., *T. simmilieri* Beauverd, *T. violacea* Harv. (tribe Tulbaghieae), *Gilliesia graminea* Lindl., *Miersia chilensis,* and *Solaria miersiodes* (tribe Gilliesieae) were selected as outgroup taxa. Voucher information of all species used in the study, and Genbank accession numbers are listed in Table 1. In order to test the monophyly of the tribes, and at species level within the Leucocoryneae, we used only the ITS region, for which we included 75 sequences generated in this project. Furthermore, we generated a total of 52 *ndhF* and 47 *matK* sequences that were included in the individual and combined phylogenetic analyses.

2.3. Phylogenetic analyses

Editing and assembling of sequences were conducted using Chromas Pro version 1.34 (Technelysium Pty, Ltd, Tewantin, Australia). Quality of sequences was assessed by visual inspection of the chromatograms. Sequences were aligned using MUSCLE integrated to MEGA5 (Tamura et al., 2011) and then revised and edited manually using BioEdit (Hall, 1999). The results from different phylogenetic inference methods were compared with reconstructed trees using both parsimony and Bayesian approaches. Analyses of individual DNA regions were conducted to explore phylogenetic signals and phylogenetic incongruences among data sets. Parsimony analyses were conducted using TNT ver. 1.1 (Goloboff et al., 2008). Heuristic searches with 10,000 random addition sequences, Tree Bisection Reconnection (TBR) swapping and holding 10 trees per replicate were performed. All optimal trees were submitted to a new round of TBR branch swapping to completion. The resulting trees were submitted to 100 cycles of Ratchet (Nixon, 1999) and Drift (both default settings). The strict consensus trees obtained from both the heuristic and new technologies searches were identical, consequently, further searches were purged. Bootstrap support (Felsenstein, 1985) was calculated running 10,000 replicates, each replica starting with a single Wagner tree, swapped with TBR, and holding a single optimal tree.

Bayesian inference was performed using MrBayes v.3.2.6 (Huelsenbeck and Ronquist, 2001). Models of molecular evolution were calculated using JModeltest (Posada, 2008) for each dataset. Three independent runs were completed to ensure that the analyses converged on the optimal tree set. Each analysis implemented four simultaneous chains and ran for 1×10^{10} generations. Tree space was sampled every 100th generation for a total sample of 10,000 trees per analysis. Each independent run reached stationary prior to the 20,000th generation. All output searches were analyzed with TRACER to determine convergence, and we discarded the first 25% of trees as burn-

in. All Bayesian and modeltests analyses were performed via the CIPRES Gateway (Miller et al., 2010). Consensus trees were visualized and edited using FigTree 1.4.3 (Rambaut, 2009). Final figures were edited using Inkscape v. 0.92 [free open-source SVG graphics editor; (Tavmjong, 2011)].

In order to detect evolutionary scenarios, such as hybridization, gene duplication, horizontal gene transfer, among others, that usually are not well represented in tree-like topologies, we used a phylogenetic network graphic (Neighbor-Net) as proposed by Hudson and Bryant (2006). The combined matrix was used as input and the analyses were performed using uncorrectedP distance, implemented in SplitsTree4 v 4.11.3 (Hudson and Bryant 2006). Bootstrap resampling was calculated using 1000 replicates. In order to complement the Neighbor-Net graph, and to evaluate reticulation in an evolutionary framework, a reticulate consensus network was also performed to reconcile different marker topologies as implemented in SplitsTree4 v 4.11.3. The complete data matrices used, and phylogenetic trees and networks are available upon request from the senior author.

2.4. Analyses of phenological overlap

More than 500 herbarium specimens of *Nothoscordum* Sect. *Nothoscordum,* and *Beauverdia* as defined by Guaglianone (1972) and Sassone et al. (2014b) respectively, were studied to document flowering time. Voucher specimens are stored at BA, BAA; BAF, BAB, MVM, MVFA and SI herbaria [acronyms follow Thiers (2017, cont. updated)]. In all, 195 specimens were used to calculate monthly flowering frequencies. Taxonomic classification and frequence of flowering time of species included are shown in Supplementary Table 1, the complete voucher information is available upon request from the senior author.

2.5. Divergence time among lineages

Calibration for the divergence time was unfeasible due to the lack of fossil records of Asparagales; hence, we used priors according to substitution rates estimated for the ITS region: a mean of 5.09×10^{-9} substitutions per site per year (sub/site/yr) following previous studies in particular, in subfamily Allioideae (Dubouzet and Shinoda, 1999; Li et al. 2016). In addition, a calibration constrain to *Allium* was estimated using a lognormal distribution on nodes with an offset value of 34.26 Ma estimated by Li et al. (2016), the standard deviation was set to 1, and the upper bound at 50.7 Ma (95% of the probable distribution). Also, tribe Leucocoryneae was set to 25 Ma offset, and upper bound at 37 Ma (95%) following Jara-Arancio et al. (2014). The topology of the initial tree was constrained to represent relationships among tribes and genera as reported in the phylogenetic results. The substitution model estimated for ITS was GTR + G + I, and searches run under the uncorrelated lognormal relaxed clock for the model tree, with the Yule process as the speciation model selected. Four independent runs were performed in BEAST 1.8 (Drummond and Rambaut, 2007) under the CIPRES Science Gateway platform (Miller et al., 2010) with the following specifications: the most recent common ancestor (MRCA) was estimated using Bayesian Markov chain Monte Carlo (MCMC) searches with 20 million generations each and sampling, every 2000 iterations. All output searches were analyzed with TRACER to determine convergence, the effective sample size of all estimated parameters (> 200) and percentage of burn-in for tree constructions. Trees were summarized with LOGCOMBINER 1.8 and TreeAnnotator v2. 1.2 (Rambaut and Drummond, 2014), using a burn-in value of 20%. The mean node heights option was selected and the posterior probability set to 0.5. The phylogenetic trees were visualized using Figtree 1.4.3 (Rambaut, 2009) with mean ages and 95% HPDs of age estimates given on tree branches.

3. Results

3.1. Sequence data

The aligned *ITS* sequence data matrix consists of 112 accessions including outgroup species and 614 characters of which 400 characters were parsimony-informative. The analysis yielded 48 most parsimonious trees of length = 1204 steps (consistency index, CI = 0.542; retention index, RI = 0.905; tree not shown). The plastid data matrix (cpDNA: *ndhF* + *matK*) consisted of a partial sampling with 76 accessions including the outgroup species, and 3135 characters of which 127 were parsimony-informative. The parsimony analysis yielded 423 most parsimonious trees of length = 1459 steps (CI = 0.72; RI = 0.92; Fig. S1). Analyses of individual regions produced similar topologies and did not reveal incongruences. Hence, ITS and plastid markers were combined in a matrix that resulted in 3398 characters of which 617 were parsimony-informative. We found 37 most parsimonious trees of length = 2501 steps (CI = 0.61; RI = 0.9). To perform Bayesian Inference analysis, the best-fit substitution model was selected for each region independently. The best-fit substitution model found for the ITS region was GTR + I + G, while for both plastid regions, a similar model was selected: GTR + G.

3.2. Phylogenetic analyses

Because both, Bayesian inference (BI) and maximum parsimony (MP) analyses yielded similar clades, we present the results based on similar topologies. It should be noted that no major discrepancies between topologies were revealed when comparing BI with MP (Fig. 1, Fig. S1 and Fig. S2). Foremost, tribe Leucocoryneae resolved as monophyletic in the independent ITS and plastid data sets as well as the combined analyses (PP: 1.0/BS: 99). The phylogenetic relationships among major tribes were examined including a complete sampling of the ITS sequence data (Fig. S1). Both South American tribes were related in a major clade, the Major South American clade (MSA, PP: 1.0/BS: 100; Fig. 1), i.e., Gilliesieae, is clearly the sister clade to Leucocoryneae; (PP: 1.0/BS: 100). Also, the MSA clade was related to the African tribe Tulbaghiae, while Allieae is sister to the clade formed by both the African tribe Tulbalghieae plus the MSA (Fig. S1).

Within the tribe Leucocoryneae, *Leucocoryne* and *Latace* are included in a clade (PP: 0.99/BS: -) and appear as the syster of the rest of the tribe. Our sampling encompasses a comprehensive number of species of *Tristagma* as well as all *Ipheion* species in both, plastid and nuclear datasets. ITS also includes additional vouchers to test the monophyly of the species (Table 1). A significant result shows *Ipheion* as monophyletic in all plastid, nuclear and combined analyses (PP: 1.0/BS: 100, Fig. 1 and S1). However, some discrepancies were found among datasets when defining relationships between *Ipheion* and *Tristagma.* Both genera are sister to each other by the plastid data analysis (Fig. S2), while the inclusion of *Ipheion* in *Tristagma* is determined with the analyses of the combined dataset and of the nuclear data alone. However, when exploring network analysis, *Ipheion* and *Tristagma* are recovered as different lineages (Fig. 2A). When exploring reticulate consensus network, conflicting signals among different dataset are recovered suggesting reticulate patterns (Fig. 2B). The ITS phylogeny shows *T. patagonicum* (Baker) Traub as the sister taxa to *Ipheion* (Fig. S1), while when combining plastid and nuclear data, a clade with *T. ameghinoi* (Speg.) Speg, *T. bivalve* (Hook. ex Lindl.) Traub, and *T. circinatum* (Sandw.) Traub appeared as sister to *Ipheion* (Fig. 1). The monophyly of all species of *Ipheion* has been confirmed in all analyses (Figs. 1, S1 and S2). However, when defining the position of species of *Tristagma*, some discrepancies were found among datasets. For instance, the phylogenetic position of *T. violaceum* (Poepp.) Traub. is inconsistent among regions; sometimes this species is nested in *T. bivalve* clade (Fig. S1) or related to *T. gracile* (Phil.) Traub, *T. nivale* Poepp. and *T. graminifolium* (Phil.) Ravenna (Fig. 1). Species of *T. circinatum*, *T. gracile, T.*

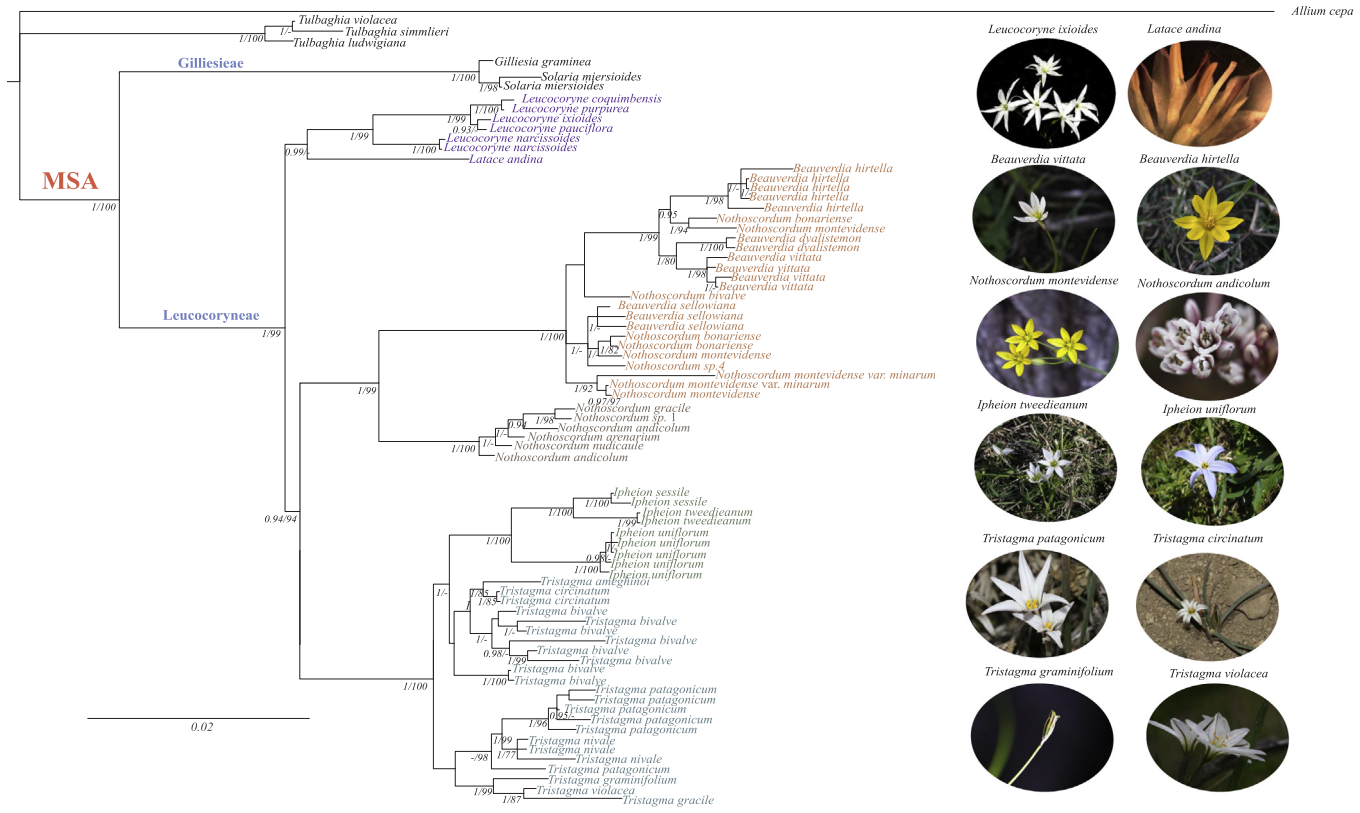

Fig. 1. Bayesian inference tree of Leucocoryneae based on a combined data using sequences of ITS region and two plastid genes: matK and ndhF. Bootstrap values ≥80% and posterior probability values ≥0.8 are indicated below branches. MSA: Major South American clade (see text for reference).

graminifolium, T. nivale, and *T. patagonicum* are monophyletic (Fig. S1).

In turn, the genus *Nothoscordum* would be monophyletic if species of *Beauverdia* are included (PP: 1.0/BS: 99; Fig. 1 and S1), both species groups are congruent with the sectional treatment as preiously proposed by Guaglianone (1972): *N.* Sect. *Inodorum* (PP: 1.0/BS: 100), and a second group of species of Sect. *Nothoscordum* plus three species of

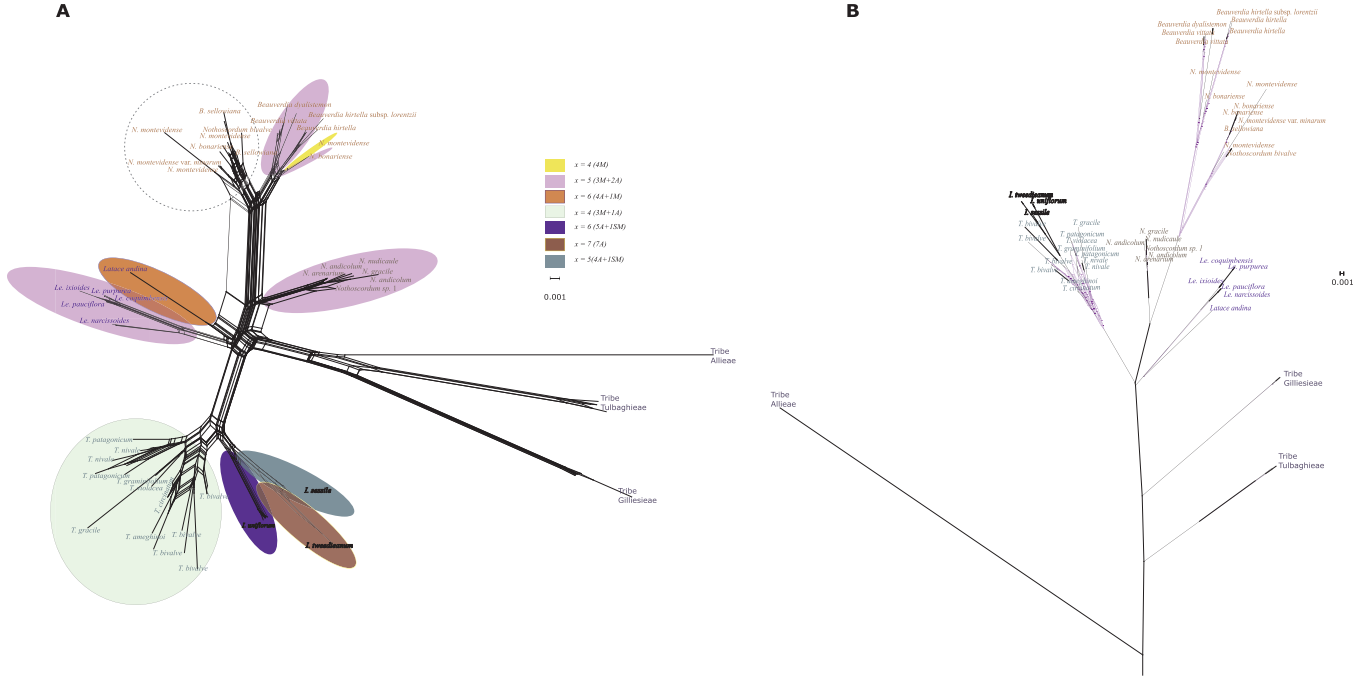

Fig. 2. A. Neighbor-Net split graph of Leucocoryneae species based on combined ITS and two plastid regions. Edge lengths are proportional to the uncorrected p-distances. Colored shades indicate base chromosome numbers. To simplify, taxon names were condensed when appearing in closed nodes. Dotted line indicates diffierent base chromosome number for species included in the circle. B. Reticulate consensus network derived from majority consensus(> 50%) trees resulting from analyses of cpDNA and equivalent ITS matrix (76 terminals) showing several instances of reticulate events postulated to reconcile discordant patterns among trees (purple lines). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Beauverdia (PP: 1.0/BS: 100). Within Sect. *Nothoscordum* + *Beauverdia* clade, a well-supported subclade gathers *B. dyalistemon* (Guagl.) Sassone & Guagl., *B. hirtella* (Kunth) Herter, *and B. vittata* (Griseb.) Herter, one specimen of *N. montevidense* Beauverd var. *montevidense* and one specimen of *N. bonariense* Beauverd, (PP: 1.0/BS: 99). While *N. bivalve*, and all other specimens of *N. bonariense,* both varieties of *N. montevidense,* and all specimens of *B. sellowiana* resolved related in a trychotomy, sister to the latter subclade (Fig. 1). However, when analysing the ITS dataset alone, two subclades are well supported in all the analyses: the Sect. *Nothoscordum* + *Beauverdia* clade, and a clade with *N. bivalve*, *N. bonariense,* both varieties of *N. montevidense*, and *B. sellowiana* (Fig. S1). The monophyly of all species of *Beauverdia* is herein confirmed with strong support (Fig. 1).

Phylogenetic network analyses were performed using the combined dataset (ITS plus *ndhF* and *matK*) including all taxa of Leucocoryneae (Fig. 2). The relationships among tribes of the subfamily Allioideae and among genera of tribe Leucocoryneae were, in general, in agreement with the MP and BI analyses. However, a reticulate evolutionary pattern possibly representing ancestral reticulation among groups was revealed by the Neighbor-Net analysis (Fig. 2A) and by the reticulate network (Fig. 2B). Specifically, species of *Nothoscordum* Sect. *Nothoscordum* and *Beauverdia* are related by a narrowly-meshed network suggesting a more complex evolutionary history than a simple ancestor-descendent natural history. Moreover, *Beauverdia sellowiana* is grouped with specimens of Sect. *Nothoscordum* in a lineage distant to the rest of *Beauverdia* species.

3.3. Analysis of phenological overlap

Because of the reticulate pattern found among species of *Nothoscordum* Sect. *Nothoscordum* and *Beauverdia*, we analyzed the flowering time as a driving hybridization factor or an isolating introgression barrier. The collection includes a total of 195 individuals with date (month) of flowering. This information was used to calculate frequencies of flowering time by month per individual species (Fig. 3). All species analyzed of Sect. *Nothoscordum* flower twice a year in early autumn and spring; however, the species exhibit a maximum frequency in one of the two seasons (Fig. 3). In contrast, three species of *Beauverdia* flower only once: *B. hirtella* (including both varieties) and *B. vittata* bloom in autumn, whereas *B. dialystemon* blooms in spring. Only *B. sellowiana* displays an extended flowering period from April to September. Considering the overlap of flowering, species of Sect. *Nothoscordum* and *Beauverdia* bloom in the same season but other species do not exhibit overlap in the flowering month (Fig. 3).

3.4. Divergence time estimation

Within the Allioideae, major lineages possibly arose about 37–32 Ma (Late Eocene to Late Oligocene), when an ancestor diverged into two lineages: the South African Tulbalghiae and the South American Gilliesieae + Leucocoryneae (MSA) (Fig. 4). During the early Miocene, tribe Leucocoryneae split into three major clades: the Chilean-Andean *Latace* + *Leucocoryne* clade (node age: 20.9 Ma; 95% HPD: 29.8–10 Ma), the *Nothoscordum* + *Beauverdia* clade (node age: 21.94 Ma; 95% HPD: 28.44–15.2 Ma), and the Chilean-Andean-Patagonian-Pampean *Tristagma* + *Ipheion* clade (node age: 20.66 Ma; 95% HPD: 27.8–13.5 Ma). Later on, and during Middle Miocene, another divergence event took place leading to the origin of new lineages, namely the Pampean genus *Ipheion* separated from *Tristagma* (node age: 12.9 Ma; 95% HPD: 20.1–6.6 Ma). At the other side, *Beauverdia*, a clade composed by yellow-white uni-flowered inflorescences, and *Nothoscordum* Sect. *Nothoscordum* (all with white-cream-yellow pluri-flowered inflorescences) separated from the crown *Nothoscordum* Sect. *Inodorum* (all with white pluri-flowered inflorescences; node age at about 17.62 Ma (95% HPD: 24.54–11.75 Ma) (Fig. 4).

4. Discussion

Morphological and cytogenetic variation within subfamily Allioideae has been extensively studied (e.g. Rudall et al., 2002; Escobar, 2012; Souza, 2012; Souza et al., 2015; Pellicer et al., 2017; Peruzzi et al., 2017). Consequently, different circumscriptions of tribes, subtribes, genera, and species have been proposed accompanied by a nomenclatural confusion, e.g. genus *Tristagma* (Arroyo-Leuenberger and Sassone, 2016). Notwithstanding previous phylogenetic inferences, our results strongly support four tribes of the monophyletic subfamily Allioideae. In fact, the Allieae is the basal tribe characterized by morphological synapomorphies such as a gynobasic style, reduced number of ovules (Rudall et al. 2002, Friesen et al., 2006), and specific cytogenetic features (Peruzzi et al., 2017). The South African tribe Tulbaghieae, sister tribe of the MSA (Fig. 1 and S1), can be easily discriminated from the other tribes within Allioideae, not only by molecular characters (Stafford and Rønsted, 2015), but also by the presence of corona in the flowers and cytogenetic traits (Vosa, 2000, 2007). The MSA includes two divergent clades (Fig. 1 and S1): the tribe Gilliesieae characterized by unique morphological characters such as zygomorphic flowers (Rudall et al., 2002; Escobar, 2012) and the tribe Leucocoryneae, characterized by actinomorphic flowers and septal nectaries (Sassone et al., 2014a). In all, our data revealed major lineages within Leucocoryneae and defined species boundaries based on cytogenetic, morphological and/or geographical data. As stated by Welles and Ellstrand (2016), we believe that hybridization, polyploidization, and Robertsonian translocations (fusion and fission of chromosomes) have been important drivers of plant evolution. In fact, these events have been used to explain high diversification rates within the Leucocoryneae (Souza et al., 2016; Pellicer et al., 2017; Sassone et al., 2018).

4.1. Clade I: Leucocoryne + Latace.

The genus *Leucocoryne*, with almost 20 species, is adapted to sclerophyllous and desert biomes on the western side of the Chilean Andes (Jara-Arancio et al., 2014). *Latace* is composed of only two species: *L. serenense* (Ravenna) Sassone endemic to the Central coast of Chile and *L. andina* that occurs at high altitudes in both sides of the Andean region of Chile and Argentina (Sassone et al., 2015). A high morphological diversification is observed within this clade but both genera have unique morphological characters: the presence of staminodes (Zoellner, 1972; Crosa, 2004; Muñoz and Moreira, 2000) characterize the genus *Leucocoryne.* On the other hand, *Latace* is clearly recognized by the presence of purplish-red inner bulb cataphylls, cuculate tepals or tepals' apices involute, and the presence of a curved long embryo (Crosa, 2004). Both genera are differentiated also by the basic chromosome number and karyotype formula: *Latace* [$x = 6$ or 12 (2M + 4A or 4 M + 8A)]; *Leucocoryne* [$x = 5$ (3M + 2A)]. However, the circumscription of *Latace* has been extensively discussed principally based on floral similarities with *Nothoscordum* (Guaglianone, 1972, 1973; Crosa, 1975, 2004; Sassone et al., 2015; Souza et al., 2016). Our molecular analyses concur with previous results (Crosa, 2004; Souza et al., 2016; Sassone et al., 2015, 2018) and confirmed the position of the genus as an independent entity from *Nothoscordum*.

4.2. Clade II.1: Nothoscordum + Beauverdia

In agreement with previous studies (Souza et al., 2016; Pellicer et al., 2017), *Nothoscordum* is paraphyletic with *Beauverdia* nested within. The split of *Nothoscordum* in two clades matches the taxonomic division of the genus in two sections: *Nothoscordum* Sect. *Inodorum* and Sect. *Nothoscordum* (Guaglianone 1972). According to Nuñez (1990), there is a barrier to gene flow between species from these sections, and this hypothesis is supported by our molecular studies (Figs. 1, 2 and 4). *Nothoscordum* Sect. *Inodorum* has a limited range of morphological

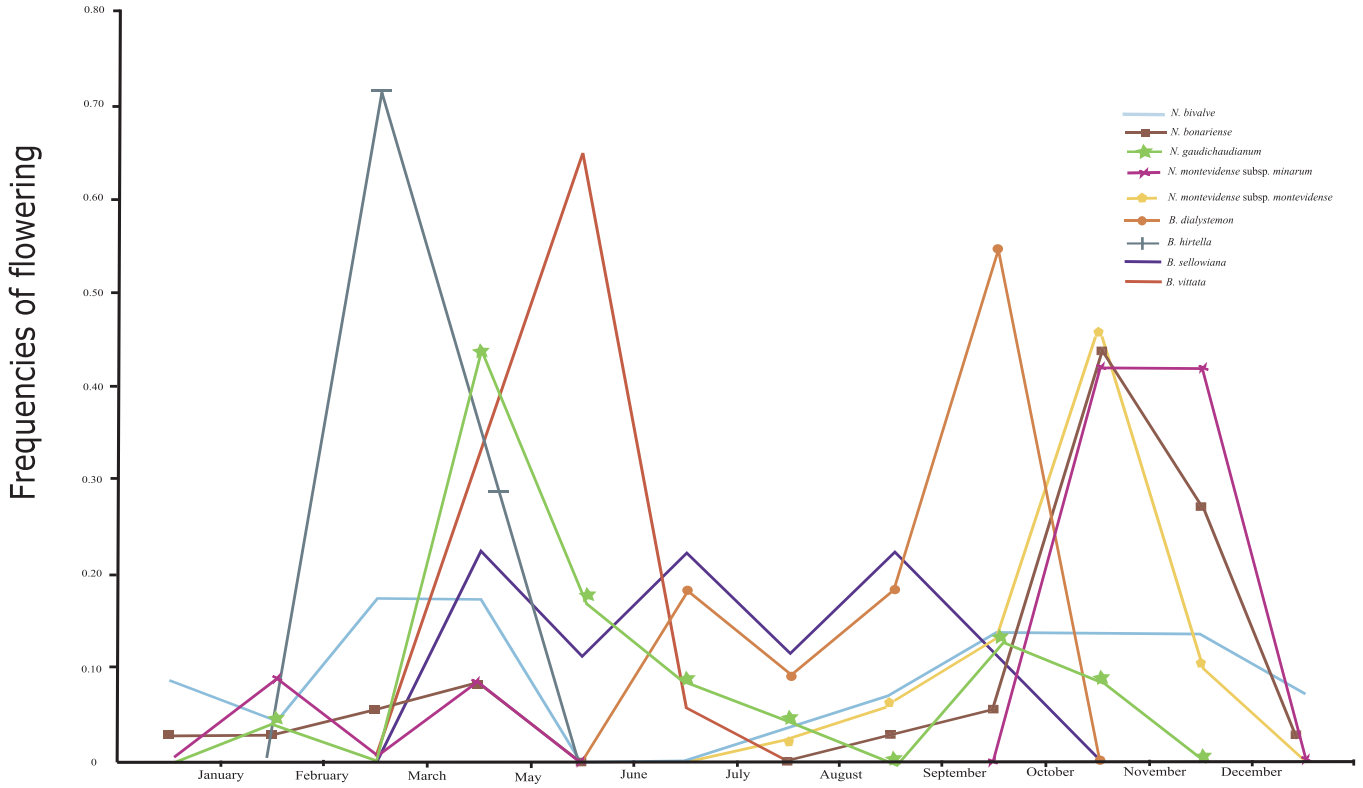

Fig. 3. Flowering frequencies time in species of *Nothoscordum* Sect. *Nothoscordum* and *Beauverdia*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

variation. The species share pluriflowered inflorescences with white flowers with staminal filaments connate at the base of the filaments. In terms of cytogenetic features, most of the species have the base chromosome number $x = 5$ and a consistent karyotype formula (3M + 2A). On the other hand, *N.* Sect. *Nothoscordum* merges with species of *Beauverdia* in all analyses (Figs. 1 and 2), a finding previously reported by Souza et al. (2016) and Pellicer et al. (2017). Although the inclusion of *Beauverdia* in *Nothoscordum* highlights the paraphyly of the genus, we believe that both lineages interbreed when species occur in sympatry indicating weak barriers to gene flow. Moreover, there are intermediate states for diagnostic morphological characters: some species have a ligule at the base of leaf blades, flowers can be white, yellow or cream, arranged to have uni- or pluriflowered inflorescences and the staminal filaments may be connate at base. Also, a high variation in karyotype features occurs within this group. For instance, $x = 4$ or 5, ploidy levels from diploid to hexaploid, different karyotype formula (4M or 3 M + 2A), and different levels of DNA content (18 to 32 pg; Pellicer et al. 2017; Sassone et al., 2018). It is worth noting that the morphology clearly differentiate *Beauverdia* from *Nothoscordum* (Sassone et al., 2013, 2014a, 2014b). Species of *Beauverdia* and the analyzed species of *Nothoscordum* Sect. *Nothoscordum* are distributed in the Pampean region from Buenos Aires, in the Mesopotamia region, and Uruguay and, to a lesser extent, they reach southern Brazil.

The phylogenetic network recovered *Nothoscordum* Sect. *Nothoscordum* + *Beauverdia* lineage with a highly reticulate pattern of relationships (Fig. 2). We hypothesize that the observed paraphyly of *Nothoscordum*, the presence of specimens of the same species in different subclades and lineages, and the reticulate structured pattern in the *Nothoscordum-Beauverdia* clade could be the result of hybridization. It is expected that closely related species could hybridize in sympatry favored by similar periods of flowering (Seehausen et al., 2014; Naciri and Linder, 2015; Kosachev et al., 2016). Species of *Nothoscordum* exhibit a higher flowering frequency in a determined season but tend to flower twice a year, facilitating mating contact with other congeneric members (Fig. 3). Our hypothesis of hybridization is enhanced by spatial overlapping populations, flowering synchrony, existence of hybrids, e.g., natural hybridization between *N. montevidense* and *N. bonariense* (Nuñez, 1990; Crosa, 2004), and a discordant pattern of cytogenetic parameters (Fig. 2A). Conversely, mixed species populations are somewhat isolated by discordant flowering time, such as *B. dialystemon* and *B. hirtella* (Fig. 3). These arguments need a verification, probably with a nuclear phylogeny of low copy nuclear genes, in which parental lineages could be tracked. Several molecular studies concluded that hybridization generates evolutionary novelties (Martin et al., 2006; Abbott et al., 2013, 2016). Furthermore, Hamilton et al. (2016) stated that hybridization is crucial to generating genetic variability assisting species to face increasing adverse environmental changes. Detailed studies, such as intercrossing species tests, are needed to corroborate natural hybridization and explain the potential paraphyly and reticulation (Hörandl and Stuessy, 2010) within the *Nothoscordum* Sect. *Nothoscordum* + *Beauverdia* clade (Figs. 1 and 2). Hybridization studies together with a comprehensive sampling of *Nothoscordum* would help to make taxonomic decisions on the circumscription of the genus including or not *Beauverdia*.

4.3. Clade II.2 *Tristagma* + *Ipheion* clade

The circumscription of *Ipheion* and *Tristagma* has been has been particularly problematic (Traub and Moldenke, 1955; Traub, 1963; Guaglianone, 1972; Ravenna, 2001b; Arroyo-Leuenberger and Sassone, 2016). Our studies recovered *Tristagma* + *Ipheion* as a strongly supported monophyletic clade (T + I clade), reinforced by morphological synapomorphies, such as tepals forming a tube always covering the ovary, stamens arranged in two-series, and flowers white, purple to light-blue (Sassone et al., 2013; Arroyo-Leuenberger and Sassone, 2016). Although the T + I clade is strongly supported and *Ipheion* is recovered as monophyletic, the position of *Ipheion* within the clade is not completely resolved. Previous molecular studies defined *Ipheion* as

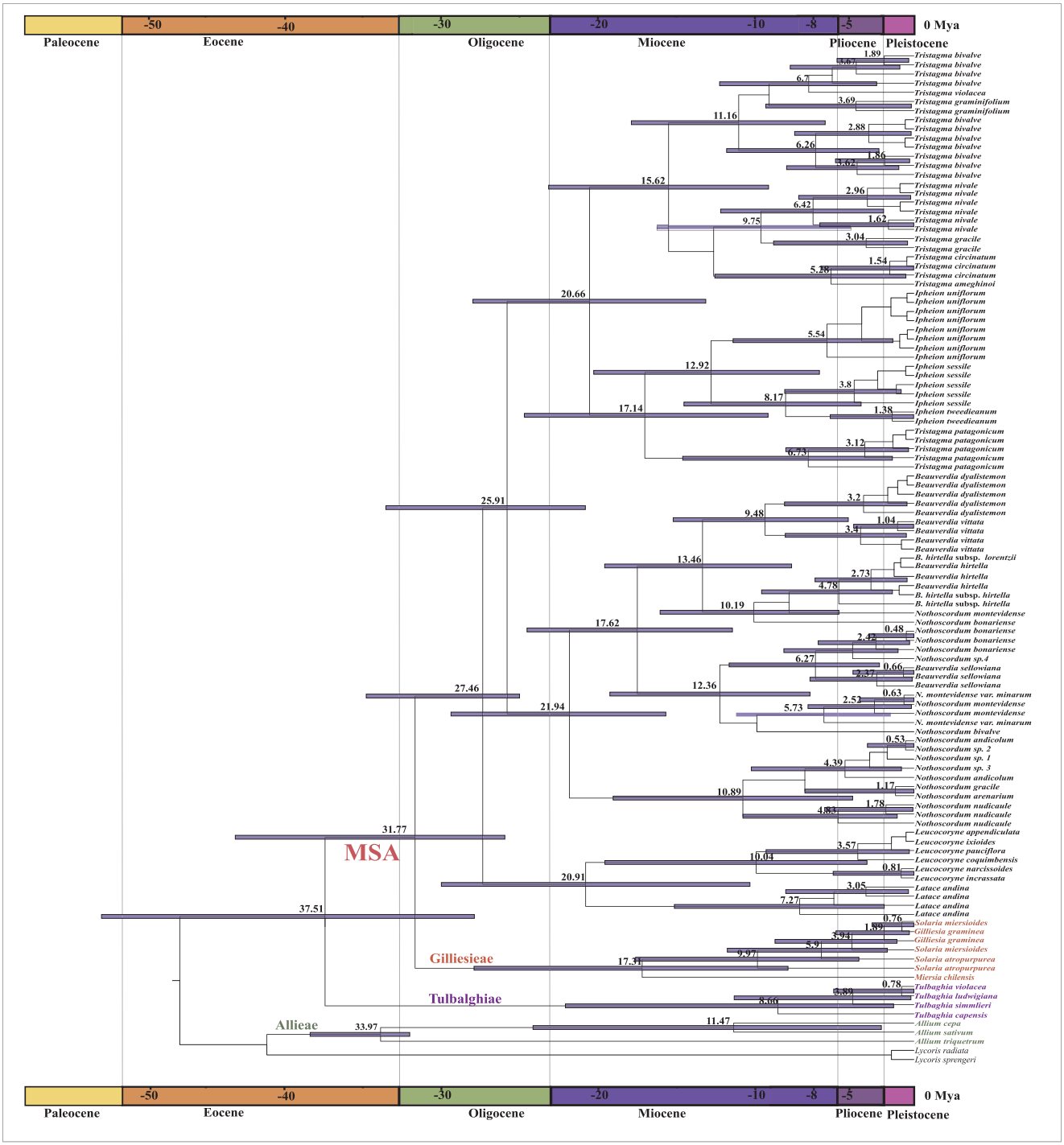

Fig. 4. Maximum clade credibility tree from the Bayesian analysis of nuclear ribosomal DNA sequences (ITS) from 112 samples representing tribes of the subfamily Allioideae, with particular emphasis in Leucocoryneae. Divergence times were inferred using a relaxed molecular clock and calibration priors based on substitution rates estimated for the ITS region by previous studies on Allioideae. Node bars represent 95% confidence intervals of divergence times. MSA: Major South American clade (see text for reference).

the sister group of *Tristagma* (Souza et al., 2016; Pellicer et al., 2017); however, ITS and the combined analyses suggest the inclusion of *Ipheion* in *Tristagma* (Fig. 1, S1), in agreement with former morphological studies (Traub, 1963; Ravenna, 2001b). Nevertheless, *Ipheion* is recognized by morphological synapomorphies such as the presence of one bifid bract, an exclusive feature within the tribe. Two other features, the presence of unifloral inflorescence and humifuse fruits are homoplastic characters shared with species of *Beauverdia* [previously treated as *Ipheion s.l.* by Guaglianone (1972)]. Furthermore, the presence of different basic chromosome numbers, karyotype formula, DNA content (summarized in Sassone et al., 2018), and the marked geographical disjunction between both genera reinforce this hypothesis (Sassone, 2017).

In this study we explored the phylogenetic relationships within *Tristagma* for the first time, including the test of the monophyly and circumscription of species using eight out of twelve species in the analyses and several representative vouchers per species. Within the *Tristagma* clade, two monophyletic subclades were found, but no major

patterns of morphological or geographical distribution correlate with those two phylogenetic cohorts. *Tristagma nivale* and *T. patagonicum* are the most widespread species (Arroyo-Leuenberger and Sassone, 2016) and several accessions included in the analysis confirmed the monophyly of both species. Lastly, the inclusion of *T. graminifolium* (= *Steinmannia graminifolia*) in the genus *Tristagma* has been somewhat controversial. This species has unique characters, such as the small size of the plant, and the presence of green, small flowers (ca. 10 mm). However, in addition to cytogenetic features (Ravenna, 1978; Sassone et al., 2018), the inclusion of this species in *Tristagma* is also supported by our molecular data (Figs. 1 and 2). *Tristagma violaceum* could be of hybrid origin; this species is suspected to be a polyploid, a hypothesis that is also supported by morphological characteristics (it is the largest *Tristagma* species) and double total DNA content within the genus (Sassone et al., 2018). Nevertheless, a major sampling of this species, chromosome counts, and the study of other loci are still needed to reinforce this hypothesis.

4.4. Divergence time estimation in Leucocoryneae and biogeographic assessments

The study of geographical disjunctions is intrinsically linked to lineage divergence. Dating divergence using molecular clocks estimations is mainly based on the accumulation of genetic differences through time (Magallón, 2004). Based on fossil calibrations and phylogenetic studies the origin of monocots has been proposed in the Cretaceous, between 134.7 and 131.6 Ma and afterward subfamilies Allioideae and Amarylloideae (Amaryllidaceae) diverged during the Eocene, at an estimated age of 55.21 Ma (Magallón et al., 2015). During this time, from the Late Paleocene to early Eocene, a warming period with a pronounced climatic optimum occurred (Zachos et al., 2001) possibly favoring the diversification of major lineages of Allioideae in the northern continents. Based on phylogenetic analyses of ITS, Dubouzet and Shinoda (1999) estimated the divergence between Old World and New World *Allium* between 50 and 25 Ma. However, using a calibrated *rbc*L phylogeny of Asparagales, a recent estimation of the origin of *Allium* constrained the origin to ca. 34.26 Ma (ca. 46–25 Ma) as reported by Li et al. (2016). The ancestor of *Allium* is believed to have its origin in the northern hemisphere (Dubouzet and Shinoda, 1999); while an Allioid lineage should have migrated towards warmer areas of southern hemisphere after the Eocene climatic optimum when the earth's climate changed to cooler conditions during 50–34 Ma (Zachos et al., 2001). It is possible that an African Allioid lineage (de Wilde –Duyfjes, 1976; Dubouzet and Shinoda, 1999) may have given rise to the southern Tulbaghieae, from which the MSA lineage diverged in South America. We hypothesize that a southern seed dispersal event from Africa to South America occurred during the Late Oligocene.

According to our results, Leucocoryneae might have originated in southern South America around 28 (35–25) Ma during Late Oligocene (Fig. 4), while tribe Gilliesieae diverged during the Miocene ca. 18 (29–7) Ma. During this period of time a warming phase, with a peak in 17–15 Ma, caused the global ice decline and a tectonic reorganization produced the uplift of the Andes in South America (Zachos et al. 2001). During the mid-Miocene to Pliocene, the uplift of the Andes has been one of the major changes promoting the evolution of the regional biota by modeling the formation of different environments in southern South America (Ortiz-Jaureguizar and Cladera, 2006; Luebert and Weigend, 2014). The diversification of Gilliesieae is also associated with the Andes formation. Right after the origin of tribe Leucocoryneae, three major lineages diverged during the Early Miocene: *Leucocoryne* + *Latace* clade diverged and diversified in Andean and Chilean landscapes [20.9 (29–10) Ma]; then, two related lineages segregated: the *Nothoscordum* + *Beauverdia* ancestor ca. 22 Ma (28–15) and the *Tristagma* + *Ipheion* ancestor at 20.6 Ma (28–13). The ancestral lineages should have been well dispersed in southern South America until a series of Atlantic marine transgressions known as "Paranean sea" during the Late Miocene affected eastern Argentina and western Uruguay in the southernmost region of the continent (Donato et al., 2003; Ortiz-Jaureguizar and Cladera, 2006). In fact, a vicariant event is particularly evident between *Tristagma* and *Ipheion* during Late to Middle Miocene when marine transgressions formed a barrier promoting geographic isolation. Moreover, several lineages diversified during Middle Miocene around 15–10 Ma such as the ancestors of *Nothoscordum* Sect. *Inodorum*, *Nothoscordum* and *Beauverdia* clade, and several genera as *Leucocoryne*, *Ipheion* and major lineages of *Tristagma* (Fig. 4). Overall, diversification of major lineages should have been favored by a period of optimum climatic conditions during the mid-Miocene (Zachos et al., 2001). After the marine transgressions in the Late Miocene to Early Pliocene (ca. 11–3 Ma), and during the period known as "the Age of the Southern Plains" (Ortiz-Jaureguizar, 1998), new habitats were available in northern Patagonia, central and northern Argentina and Uruguay. Therefore, new suitable environments after the Andean uplift and during the Age of the Southern Plains provided a favorable scenario for radiation and speciation of major extant lineages of the Leucocoryneae in the southern Pampas, extra-Andean Patagonia, Andean mountains, and in Chile.

5. Conclusions

Phylogenetic relationships within the tribe Leucocoryneae are partially explained by ancestor-descendent phylogenetic trees, and also by a complex interaction network among taxa, revealing a reticulate pattern of relationships. In particular, together with the study of flowering time and the inclusion of previous morphological and cytogenetic studies, the hypothesis of hybridization between species of *Nothoscordum* and *Beauverdia* is strongly reinforced. Diversification in Leucocoryneae is primarily associated with Robertsonian translocations and allopolyploidy events. The crown estimated divergence time for the tribe Leucocoryneae and allied tribes of subfamily Allioideae is set in the Oligocene which concurs with the evolution of species and lineages in conjunction with past climatic changes. We posit that the uplift of the Andes, flooded lands as vicariant events, and subsequent marine transgressions led to the diversification of lineages in South America, areas in which different genera radiated and colonized new environments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ympev.2018.04.034.

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