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Genome size dynamics in tribe Gilliesieae (Amaryllidaceae, subfamily Allioideae) in the context of polyploidy and unusual incidence of Robertsonian translocations

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This study focuses on tribe Gilliesieae (Amaryllidaceae, Allioideae), which stand out because of their karyotype diversity, constituting a textbook example of Robertsonian translocations (RTs), in which chromosomes fuse or break at the centromere. Polyploidy (i.e. whole genome duplication, WGD) is also common in the tribe, hence making Gilliesieae particularly suitable for investigating two major processes of genome evolution in an integrated way. Our phylogenetic reconstruction supported a two subtribe classification, Gilliesiinae and Leucocoryninae, the latter taxonomically validated in this paper. Leucocoryninae are composed of three well-supported lineages, corresponding to the genera *Leucocoryne* + *Latace*, blue-flowered (typical) *Ipheion* + *Tristagma* and *Nothoscordum* (including yellow-flowered *Ipheion* = *Beauverdia*). Analysis of the chromosome data in Leucocoryninae indicates that WGDs have resulted in an almost proportional genome size (GS) increase in *Leucocoryne*, in contrast to the reduction in monoploid GS in polyploid *Nothoscordum*. Likewise, contrasting patterns of GS dynamics and extraordinary karyotype diversity have been recovered in *Ipheion*, *Tristagma* and *Nothoscordum*, clearly illustrating the impact of RTs in shaping genome evolution in these plants.

ADDITIONAL KEYWORDS: ancestral trait reconstruction – C-value – fundamental chromosome number – karyotype – phylogeny.

INTRODUCTION

Amaryllidaceae *sensu* APG IV (2016) belong to Asparagales and consist of three subfamilies: Amaryllidoideae (e.g. amaryllises, clivias, daffodils), Agapanthoideae (African bluebells) and Allioideae (e.g. chives, garlics, onions). The phylogenetic relationships between the three subfamilies have been extensively investigated (Fay & Chase, 1996; Meerow *et al.*, 1999; Fay *et al.*, 2000; Givnish *et al.*, 2006; Pires *et al.*, 2006; Seberg *et al.*, 2012), often placing Amaryllidoideae and Allioideae as sister lineages, with Agapanthoideae as sister to both, although this relationship is weakly supported in most studies.

Allioideae consist of *c*. 14 genera and *c*. 600–700 species of rhizomatous or bulbous geophytes that can be recognized by their umbel-type inflorescences subtended by two bracts that enclose the inflorescence in a bud, a superior ovary, a solid style and frequent occurrence of thiosulphinates, including allicin (Block, 1985). Fay & Chase (1996) found that the former tribe Brodiaeeae of Alliaceae *s.s.* were more closely related to families now placed in a broader Asparagaceae (APG

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IV, 2016). This lineage is therefore not discussed here, even though it was previously included in Alliaceae (e.g. Dahlgren, Clifford & Yeo, 1985), and some species of *Leucocoryne* Lindl. were originally described as members of *Brodiaea* Sm. or *Triteleia* Douglas ex Lindl.

Amaryllidaceae (as Alliaceae) were segregated into three subfamilies by Fay & Chase (1996): Allioideae, Gilliesioideae and Tulbaghioideae, with Allioideae sister to the other two subfamilies. However, since Alliaceae are now treated as a subfamily of Amaryllidaceae (APG IV, 2016), the taxonomic ranking of these subfamilies have been transferred to the tribe level: that is, Allieae, Gilliesieae and Tulbaghieae. Gilliesieae are bulbous herbs with a concentration of species in temperate South America. They have traditionally been divided into two main lineages, previously treated as tribes, but here treated as subtribes, namely Gilliesiinae and Leucocoryninae, with zygomorphic and actinomorphic flowers, respectively (Fig. 1; Rudall *et al.*, 2002). Leucocoryninae are nomenclaturally validated here: Leucocoryninae M.F.Fay & Christenh. subtrib. nov., and transferred from the full and direct reference to the type and Latin description of Leucocoryneae Ravenna (*Onira* 5: 43. 2001: 43). Members of subtribe Leucocoryninae have a complex taxonomic history and the generic delimitation has been problematic in the past and continues to be so. These plants have linear leaves and scapose umbellate inflorescences sometimes reduced to a single flower (in *Ipheion* Raf.), with petaloid tepals often presenting a venation contrasting with the background colour (Fig. 1), which makes several of the species popular as garden ornamentals. Most commonly found in the bulb trade is *Ipheion uniflorum* Raf. (Fig. 1D, E), known by the common name of 'spring starflower'. There are numerous cultivars, including 'Rolf Fiedler' that has been suggested to be a possible separate species (Castillo, 1986). Certainly, nomenclatural instability in Leucocoryninae has long plagued the horticultural trade. For example, *I. uniflorum* has been treated under *Beauverdia* Herter, *Brodiaea*, *Hookera* Salisb., *Leucocoryne*, *Milla* Cav., *Tristagma* Poepp. and *Triteleia* and some species have been more or less randomly placed in *Nothoscordum* Poepp. or *Tristagma* (Castillo, 1986). *Nothoscordum fragrans* Kunth has been included in *Allium* L., *Geboscon* Raf., *Hesperocles* Salisb., *Maligia* Raf., *Milla*, *Ornithogalum* L. and *Sowerbaea* Sm. (Rudall *et al.*, 2002; Fay & Chase, 2006; Fay, Rudall & Chase, 2006).

Past efforts using morphological and DNA sequence data have firmly confirmed the above-mentioned subdivision of Gilliesieae into two subtribes (Fay *et al.*, 2006). The latest revision of Gilliesiinae (Escobar, 2012) accepted the genera *Ancrumia* Harv. ex Baker, *Gethyum* Phil., *Gilliesia* Lindl., *Miersia* Lindl., *Schickendantziella* Speg., *Solaria* Phil., *Speea* Loes. and *Trichlora* Baker. The most recent circumscription of Leucocoryninae encompasses the genera *Beauverdia*, *Ipheion*, *Latace* Phil. [synonym *Zoellnerallium* Crosa (1975)], *Leucocoryne* (synonyms *Pabellonia* Quezada & Martic. and *Stemmatium* Phil.), *Nothoscordum* and *Tristagma* (Sassone, Arroyo-Leuenberger & Giussani, 2014; Sassone, Belgrano & Guaglianone, 2015). In the absence of apparent differences in floral morphology (Fig. 1), efforts have been made to differentiate between *Ipheion*, *Nothoscordum* and *Tristagma* by using subjectively delimited, continuously or discretely variable morphological character states, such as the degree of fusion of the tepals and the number of flowers per inflorescence (Guaglianone, 1972; Rahn, 1998). This often resulted in an artificial circumscription and classification of these genera, not necessarily reflecting evolutionary relationships within and between these genera. For example, in Leucocoryninae all species with one flower per inflorescence were previously placed in *Ipheion*. However, this character is now known to be homoplasious in the subtribe (Fay *et al.*, 2006; as 'Ipheieae').

Robertsonian translocation (RT) events, which involve either metacentric chromosome fission at the centromere to create two acrocentric chromosomes or fusion of two acrocentric chromosomes leading to a metacentric chromosome, are the most important type of rearrangements governing chromosomal evolution in animals, but are relatively uncommon in higher plants (Jones, 1998; Leitch *et al.*, 2010). Nonetheless, they have been reported frequently in Gilliesieae (Levan & Emswellers, 1938) and RT in *Nothoscordum* has been used as a classic example in the literature (Jones, 1998). Numerous studies have focused on different representatives of Gilliesieae (see Souza, 2012), leading to the identification of several bimodal karyotypes in the tribe, with metacentric chromosomes that are approximately twice as long as acrocentric chromosomes (Goldblatt, 1976; Jones, 1998; Souza *et al.*, 2009, 2016; Escobar, 2012). In addition, such studies have also provided evidence of the role that polyploidy has played in the evolution of some clades (e.g. Escobar, 2012; Souza, Crosa & Guerra, 2015). Gilliesieae therefore provide an ideal subject for an integrated investigation of how these two cytogenetic processes of RT and polyploidy can impact genome evolution.

Chromosomal evolution is intimately linked to changes in genome size (GS) and this trait has been shown to have a considerable impact on many aspects of the phenotype, ecology and evolution of plants (e.g. Greilhuber & Leitch, 2013). Evidence suggests that the vast majority of angiosperms have small genomes (1C ≤ 2.6 pg; Leitch *et al.*, 2010), the 1C-value being the amount of DNA in the nucleus of an unreplicated

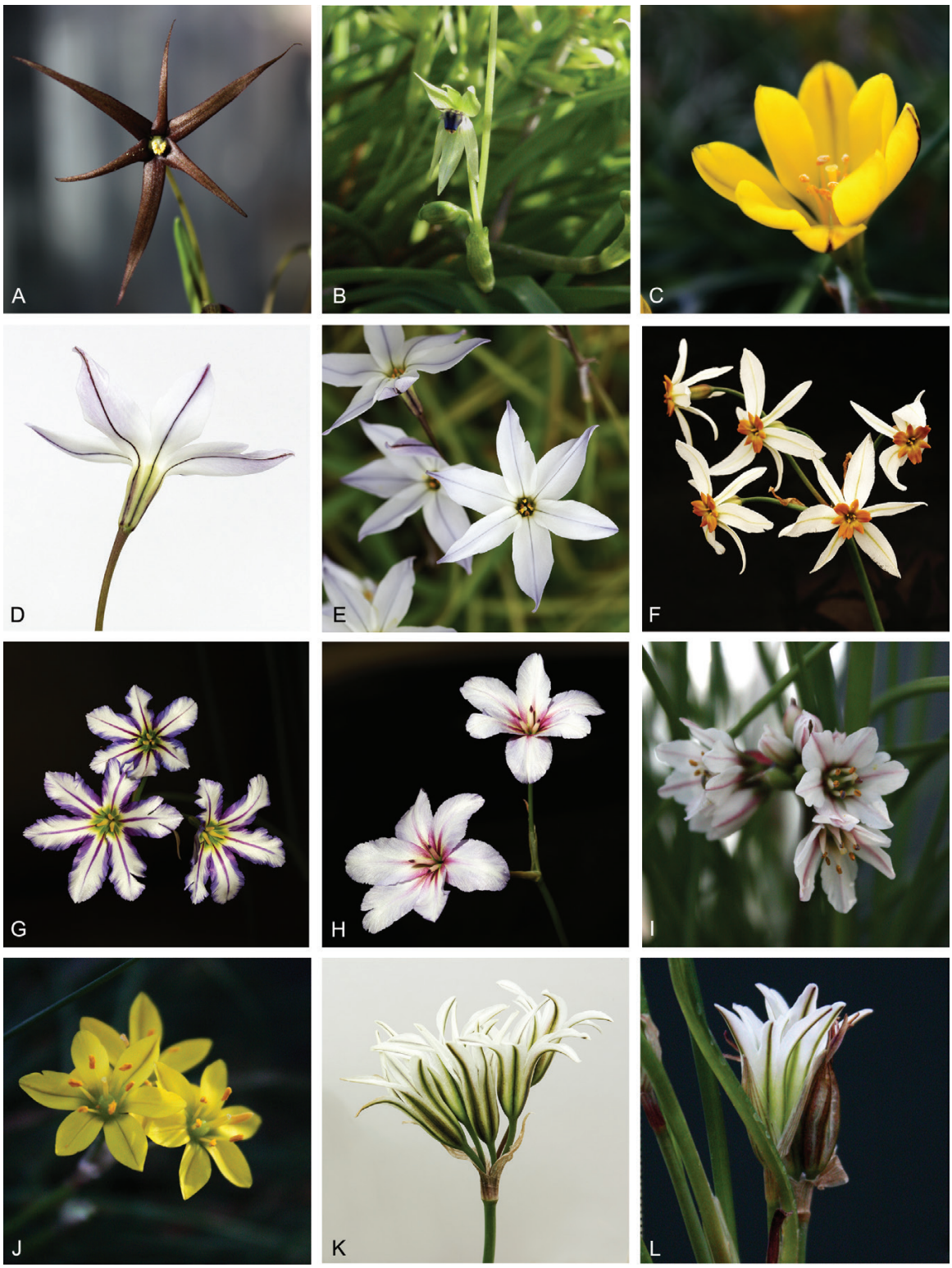

Figure 1. Floral diversity in specimens of Gilliesieae from the living collection of the Royal Botanic Gardens, Kew. Gilliesiinae. (A) *Gethyum atropurpureum*, (B) *Miersia chilensis*. Leucocoryninae. (C) *Ipheion dialystemon*, (D, E) *Ipheion uniflorum*, (F) *Leucocoryne narcissoides*, (G) *Leucocoryne pauciflora*, (H) *Leucocoryne purpurea*, (I) *Nothoscordum andicolum*, (J) *Nothoscordum montevidense*, (K) *Tristagma bivalve*, (L) *Tristagma porrifolium*. [Photographs are, copyright © 2016, by Gorm Shackelford (D, E, F, G, H, K) and by Jaume Pellicer (A, B, C, I, J, L)].

gamete (Greilhuber *et al.*, 2005). Notwithstanding, a huge range of GSs has been encountered, spanning nearly 2400-fold (Leitch & Leitch, 2013). Large genomes have primarily been observed in monocots, with the largest values recorded so far in *Paris japonica* Franch. (Melanthiaceae, 1C = 152.2 pg; Pellicer, Fay & Leitch, 2010). Such diversity has been underpinned by polyploidy, whereby plants have more than two complete sets of chromosomes per cell (Leitch & Bennett, 1997) and by the amplification of non-coding repetitive DNA, such as transposable elements, present in high frequencies in plant genomes (Bennetzen & Wang, 2014). To understand better the underlying mechanisms maintaining the staggering diversity of GSs encountered in plants, it is thus necessary to continue gathering and documenting data across lineages. According to the most recent release of the Plant DNA C-values Database (Garcia *et al.*, 2014), there are GS estimates for 177 species in Allioideae, but only two belong to Gilliesieae, so it is critical to fill these taxonomic gaps to have a clear picture of the overall diversity and dynamics of GS evolution in this lineage.

Bearing in mind previous reports of significant cytogenetic diversity in the group, we have conducted a survey across the tribe gathering karyotype and GS data, complemented with a robust new phylogenetic framework to (1) understand the evolutionary relationships among representatives of the tribe better, (2) assess the extent of both chromosome and GS diversity in Gilliesieae, (3) reconstruct ancestral character states for GS and karyological data and (4) provide further insights into the main pivotal processes driving genome evolution of the group.

MATERIAL AND METHODS

Plant material

All samples studied here were obtained from plants growing in the living collections held at the Royal Botanic Gardens, Kew (RBGK). Accession numbers corresponding to the species studied are given in Table 1. Whenever possible, herbarium vouchers were prepared and deposited in the herbarium of the Royal Botanic Gardens (K). For those accessions with few adult individuals, photographic vouchers were made, which are available from the authors upon request.

Chromosome counts and karyotype analysis

Root tips were collected from the same accessions for which GS was measured. These were pretreated in 0.05% colchicine solution at room temperature for 24 h and then fixed for at least 4 h in 3:1 absolute ethanol-glacial acetic acid at room temperature, before being stored in this fixative at 4 °C for at least 24 h. For chromosome preparations, roots were removed from the fixative and rinsed in distilled water at room temperature for 1 min, hydrolyzed in 1 M HCl for 5–8 min at 60 °C and transferred to Feulgen (Schiff's) reagent at room temperature for 30 min before being squashed on a microscope slide in a drop of 45% acetic acid. The best metaphase plates were photographed with a ProgRes C14 plus digital camera (Jenoptik Corporation, Jena, Germany) mounted on an Axioplan 2 microscope (Zeiss, Oberkochen, Germany) and images were analysed with ProgRes CapturePro v.2.8.8 (Jenoptik Corporation). The chromosome number, ploidy, karyotype formula and fundamental number (FN, the number of chromosome arms) were determined on the basis of approximately five metaphase plates.

Genome size estimation by flow cytometry

Nuclear DNA contents were estimated following the one-step flow cytometry procedure described by Doležel, Greilhuber & Suda (2007). Briefly, *c*. 1 cm^2 of leaf material from both the individual being studied and the appropriate calibration standard, either *Pisum sativum* 'Ctirad' (2C = 9.09 pg) or *Allium cepa* 'Ailsa Craig' (2C = 34.89 pg) (Doležel *et al.*, 1998; Clark *et al.*, 2016), were chopped together using a new razor blade in a Petri dish containing 1 mL 'general purpose buffer' (GPB; Loureiro *et al.*, 2007) supplemented with 3% PVP-40. A further 1 mL GPB was added and the resulting suspension was filtered through a 30 µm nylon mesh and the nuclei stained with 100 µL propidium iodide (1 mg/mL). Samples were kept on ice for 15 min and the relative fluorescence of 5000 particles was then recorded using a Partec Cyflow SL3 flow cytometer (Partec GmbH, Münster, Germany) fitted with a 100 mW green solid-state laser (532 nm, Cobolt Samba, Solna, Sweden). Three leaves from different individuals were measured separately for each accession and three replicates of each leaf were processed. The output histograms were analysed with the FlowMax software v.2.4 (Partec GmbH).

Molecular phylogenetic reconstruction

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from silica-dried leaf material from 60 accessions of 25 species of Gilliesieae using the 2× CTAB method (Doyle & Doyle, 1987) with minor modifications. Samples were purified using Nucleospin DNA purification columns (QIAquick; Qiagen Ltd., Crawley, UK) according to the manufacturer's protocols. Total genomic DNA of *Tulbaghia ludwigiana* Harv. and *T. simmleri* Beauverd, used as out groups in the phylogenetic reconstruction, was

Table 1. List of species of Gilliesieae used in the present study for which nuclear DNA contents and chromosome values have been assessed

Taxa	RBGK accession number	1C-value (SD) (pg)	1C*x*-value (pg)	2*n*	Ploidy	Karyotype formula	Fundamental number*
Subtribe Gilliesiinae							
Gethyum atropurpureum F.Phil.	1988-1970	19.94 (0.09)	19.95	14	2	4M + 4SM + 6A	22
Gethyum artropurpureum	2008-1228	19.75 (0.07)	19.75	14	2	4M + 4SM + 6A	22
Gilliesia graminea Lindl.	1977-2342	19.25 (0.14)	19.25	14	2	4M + 4SM + 6A	22
Gilliesia graminea	1977-1670	19.14 (0.14)	19.14	14	2	4M + 4SM + 6A	22
Gilliesia montana Poepp. & Endl.	2008-3044	19.90 (0.07)	19.91	14	2	4M + 4SM + 6A	22
Miersia chilensis Lindl.	2003-3578	26.46 (0.29)	26.47	20	2	2M + 18A	22
Solaria miersioides F.Phil.	2008-3049	18.41 (0.12)	18.41	14	2	4M + 4SM + 6A	22
Speea humilis (Phil.) Loes. ex. K.Krause	2008-1227	31.07 (0.14)	31.07	12	2	8M + 2SM + 2A	22
Subtribe Leucocoryninae							
Ipheion dialystemon Guagl.	1984-3093	36.38 (0.27)	36.38	10	2	6M + 4A	16
Ipheion dialystemon	1989-2632	36.12 (0.29)	36.12	10	2	6M + 4A	16
Ipheion hirtellum (Kunth) Traub.	1986-3905	21.35 (0.06)	21.35	10	2	6M + 4A	16
Ipheion hirtellum	1987-3395	21.50 (0.10)	21.51	10	2	6M + 4A	16
Ipheion sessile (Phil.) Traub.	1985-2641	20.79 (0.08)	10.40	20	4	2SM + 18A	22
Ipheion uniflorum Raf.	1994-160	21.40 (0.26)	10.70	24	4	4SM + 20A	28
Ipheion uniflorum 'Rolf Fiedler'	1981-2444	20.64 (0.16)	10.32	24	4	–	–
Ipheion uniflorum 'Album'	1988-3378	10.32 (0.04)	10.32	12	2	–	–
Ipheion uniflorum 'Jessie'	1994-159	20.48 (0.11)	10.24	24	4	–	–
Ipheion uniflorum 'Violaceum'	1973-15748	10.37 (0.04)	10.37	12	2	–	–
Ipheion uniflorum 'Charlotte Bishop'	2000-2528	10.39 (0.06)	10.39	12	2	–	–
Ipheion vittatum (Griseb.) Traub.	2006-1196	19.95 (0.08)	19.95	10	2	6M + 4A	16
Leucocoryne appendiculata Phil.	2006-39	27.49 (0.11)	27.49	10†	2†	–	–
Leucocoryne coquimbensis F.Phil. ex Phil	1976-6116	60.64 (0.29)	30.32	18	4	14M + 2SM + 2A	34
Leucocoryne coquimbensis	1987-4154	61.20 (0.56)	30.60	18	4	16M + 2A	34
Leucocoryne coquimbensis	1960-67021	60.24 (0.42)	30.12	18†	4†	–	–
Leucocoryne coquimbensis	1976-3980	59.11 (0.17)	29.56	18†	4†	–	–
Leucocoryne coquimbensis	1990-857	60.00 (0.42)	30.00†	18†	4†	–	–
Leucocoryne coquimbensis	2006-38	27.87 (0.30)	27.87	10†	2†	–	–
Leucocoryne coquimbensis	1973-807	28.05 (0.29)	28.06	10	2	6M + 4A	16
Leucocoryne incrassata Phil.	2006-40	51.98 (0.15)	25.99	18†	4†	–	–
Leucocoryne ixioides Lindl.	2003-451	59.12 (0.70)	29.56	18	4	14M + 2SM + 2A	34
Leucocoryne ixioides	1973-6318	59.87 (0.12)	29.94	18	4	–	–
Leucocoryne ixioides	1992-1304	57.82 (1.15)	28.91	18†	4†	–	–
Leucocoryne ixioides	1977-2344	57.06 (1.20)	28.53	18†	4†	–	–
Leucocoryne macropetala Phil.	2006-41	58.89 (0.05)	29.45	18†	4†	–	–
Leucocoryne narcissoides Phil.	1987-4148	53.41 (0.43)	26.71	18	4	14M + 2SM + 2A	34
Leucocoryne narcissoides	1992-1051	52.46 (0.75)	26.23†	18	4†	–	–
Leucocoryne cf. *narcissoides*	1988-1966	79.32 (0.38)	26.44	26	6	22M + 4A	48
Leucocoryne odorata Lindl.	1987-3641	56.21 (0.18)	28.10	18†	4†	–	–
Leucocoryne pauciflora Phil.	1991-8016	28.74 (0.12)	28.74	10†	2†	–	–
Leucocoryne pauciflora	1977-6731	27.89 (0.14)	27.90	10	2	6M + 4A	16
Leucocoryne pauciflora	1973-793	28.17 (0.22)	28.17	10†	2†	–	–
Leucocoryne pauciflora	1977-6123	27.83 (0.11)	27.83	10†	2†	–	–
Leucocoryne pauciflora	1961-70601	27.96 (0.18)	27.96	10	2	6M + 4A	16
Leucocoryne purpurea Gay	1998-2747	59.51 (0.12)	29.76	18	4	14M + 2SM + 2A	34
Leucocoryne purpurea	2002-118	43.11 (0.26)	28.74	14	3	10M + 4A	24
Leucocoryne vittata Ravenna	2006-42	28.95 (0.11)	28.96	10†	2†	–	–
Nothoscordum andicolum Kunth.	1986-104	32.29 (0.26)	32.29	10	2	8M + 2A	18

Table 1. *Continued*

Taxa	RBGK accession number	1C-value (SD) (pg)	1C*x*-value (pg)	2*n*	Ploidy	Karyotype formula	Fundamental number*
Nothoscordum montevidense subsp. *latitepalum* (Guagl.) Ravenna	1985-2643	47.66 (0.12)	11.92	32	8	32M	64
Nothoscordum montevidense subsp. *minarum* (Beauverd) Ravenna	1976-3834	46.96 (0.22)	11.74	32	8	32M	64
Nothoscordum sp.	2003-2563	28.72 (0.14)	14.36	18	4	14M + 4A	32
Tristagma bivalve (Hook. ex Lindl.) Traub	1979-783	19.33 (0.14)	19.33	8	2	6M + 2A	14
Tristagma nivale Poepp.	1993-363	20.66 (0.11)	20.67	8	2	6M + 2A	14
Tristagma porrifolium (Poepp.) Traub	1988-1184	35.46 (0.11)	17.73	16	4	–	–
Tristagma porrifolium	1988-8211	35.47 (0.22)	17.74	16	4	12M + 4A	28

*Fundamental number (FN) = number of chromosome arms.
†Values inferred based on the results found in either close relatives or different accessions from the same species.

supplied by the DNA & Tissue Collections, RBGK (ID 17512 and ID 17513, respectively). Two plastid regions (*matK* and *ndhF*) and one nuclear region (*ITS*) were amplified for each sample. The partial *matK* region was amplified using primers XF and 5R (Ford *et al.*, 2009) or, when that was not successful, with primers 390F and 1326R (Sun, McLewin & Fay, 2001). Two overlapping parts of the *ndhF* region were amplified using the primer pairs 32F/1318R and 1101F/2110R, respectively (Pires & Sytsma, 2002). Since we encountered problems amplifying the *ndhF* region in *Miersia chilensis* Lindl. and *Speea humilis* (Phil.) Loes. ex K. Krause, a set of specific primers was designed to carry out nested and half-nested amplifications for these taxa (Appendix S1). The *ITS* region was amplified using the primers 16SE and 27SE (Sun *et al.*, 1994). PCR amplifications were conducted in 25 µL reactions, using 22.5 µL Reddy PCR Master Mix (1.5 and 2.5 mM $MgCl_2$ for nuclear and plastid markers, respectively; ABgene, Epsom, Surrey, UK), 0.5 µL TBT-PAR mixture (*sensu* Samarakoon, Wang & Alford, 2013), 0.5 µL each primer (100 ng/µL) and 1 µL template DNA. PCR conditions were as follows. For *matK* we used: initial denaturation at 94 °C for 2 min 30 s, 30 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min 30 s and a final extension at 72 °C for 7 min. For *ndhF* we followed Pires & Sytsma (2002). For *ITS* and any unsuccessful amplifications of plastid DNA regions we used: initial denaturation at 80 °C for 5 min, followed by 35 cycles at 95 °C for 1 min, 48 °C for 1 min, 65 °C for 5 min and a final extension at 65 °C for 4 min. Resulting PCR products were purified using DNA purification columns (QIAquick; Qiagen Ltd., Crawley, UK) and sequenced using the Big Dye terminator v3.1 chemistry (ABI, Warrington, Cheshire, UK) on an ABI 3730 Genetic Analyser (ABI/Hitachi, Warrington, Cheshire, UK).

Sequence editing, alignment and phylogenetic analysis

New DNA sequences and sequences downloaded from GenBank were edited using Geneious computer software version 7.1 (Biomatters Ltd., New Zealand). The data set of 96 accessions included (1) *ITS*, *ndhF* and *matK* sequences for two out group species and 60 Gilliesieae accessions from the living collection of RBGK representing 25 species, (2) *ITS*, *ndhF* and *matK* sequences from GenBank for 12 species and (3) *ITS* accessions for 22 species and subspecies from the recent study of Jara-Arancio *et al.* (2014). The plastid DNA data set was aligned manually, whereas *ITS* sequences were aligned on the Guidance web server (Penn *et al.*, 2010) using the MAFFT multiple sequence alignment algorithm (Katoh *et al.*, 2005), with 100 bootstrap repeats, a Max-Iterate of 1000 (long run) and a local pair refinement strategy. A combined Bayesian analysis was carried out with MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003) on the CIPRES server (Miller, Schwartz & Pfeiffer, 2010). The data set was partitioned by region and, for *matK* and *ndhF*, also by codon position. The most appropriate models for nucleotide substitution were chosen with jModelTest 0.1 (Guindon & Gascuel, 2003; Posada, 2008), which were GTR + G for *matK* and *ndhF* and GTR + I + G for *ITS*. For each analysis, two concurrent runs of four Markov Chain Monte Carlo (MCMC) simulations each (one cold and three heated chains with a temperature of 0.2) were processed simultaneously for 18×10^6 generations and sampled every 700 generations. The data were then evaluated for convergence using Tracer v.1.6 (Rambaut & Drummond, 2007). Data from the first 5000 generations were discarded as the 'burn-in' period. The 50% majority rule consensus tree and posterior probabilities (PPs) of nodes were calculated from the pooled samples.

Reconstruction of ancestral character states

Reconstruction of ancestral GS values

A sample of 1000 post-burn-in trees was filtered from the Bayesian phylogenetic analysis using BayesTrees v.1.3 (http://www.evolution.rdg.ac.uk/BayesTees.html) and pruned to a selection of taxa restricted to the lowest ploidy available. After log transformation, ancestral 1C*x*-values were reconstructed with BayesTraits v.2 (http://www.evolution.rdg.ac.uk/BayesTraits.html). The best-fitting model for analysis of continuous characters (i.e. directional vs. random walk) was chosen calculating the logarithm of the harmonic mean from five independent BayesFactor tests under the MCMC option. The settings used were as follows: sampling every 1000 generations, iterations = 100×10^6, burn-in = 10×10^6 iterations, scaling parameters estimated = delta (δ), kappa (κ) and lambda (λ). The directional walk model was supported in most of the runs and the posterior distributions of the scaling parameters generated were used as the settings for the second phase of the analysis in which the ancestral 1C*x* (Anc1C*x*)-values of specific nodes were estimated with the addMRCA (most recent common ancestor) command. In addition, Anc1C*x*-values were also reconstructed using maximum likelihood (ML) and maximum parsimony (MP). Character reconstruction under ML was performed using the fastAnc command and subsequently mapped onto the Bayesian consensus phylogram using the contMap command of Phytools package using R (Revell, 2012). For the MP reconstruction, we used Mesquite v.3.04 software (Maddison & Maddison, 2007).

Reconstruction of ancestral chromosome numbers

The probabilistic models of chromosome number evolution implemented in ChromEvol v.2 (Mayrose, Barker & Otto, 2010; Glick & Mayrose, 2014) were used to infer ancestral haploid (n) chromosome numbers and haploid FN in Gilliesieae. Chromosome counts were obtained from the accessions used in the present study and complemented with available data from the literature (see Appendix S2). ChromEvol software implements ten models of chromosome number change to give an estimation of the number and type of evolutionary events taking place in a phylogenetic framework. Chromosome number transitions taken into account are dysploidy (gains and losses), duplication events (i.e. polyploidy, doubling of chromosome number) and demi-duplication (e.g. the result of a fusion between two gametes that have different ploidies). All models were fitted to the data using a Bayesian inference (BI) consensus phylogram pruned to a selection of taxa with reliable chromosome data. Each model was run for 10 000 simulations with the data provided, with the maximum number of chromosomes set to ten times higher than the highest chromosome number in the data and the minimum being set to 1, with non-fixed root number. The model that best fitted the data set was chosen under the Akaike information criterion (AIC).

RESULTS

Phylogenetic reconstruction of Gilliesieae

Nucleotide sequence data have been deposited in the DDBJ/EMBL/GenBank under the following accession numbers: LT718262–LT718323 for *matK*, LT718324–LT718387 for ITS and LT718388–LT718445 for *ndhF*. The phylogenetic tree resulting from the Bayesian analysis of the combined data set is presented in Figure 2. Separate analyses of the different markers did not reveal supported incongruence, with some exceptions restricted to the clade comprising *Gilliesia* and related genera (as previously discussed in Escobar, 2012). Our results support the division of the tribe into two major clades (i.e. subtribes Gilliesiinae and Leucocoryninae). Overall, we found strong phylogenetic support for most of the major clades, but failed to support the split between the clade containing *Tristagma* and the predominantly blue-flowered *Ipheion* and the clade containing *Nothoscordum* and the yellow-flowered *Ipheion*. The addition of multiple accessions yielded no major conflicts, but in the case of *Leucocoryne* many of the species fell in an unresolved polytomy due to the lack of sequence variation in the three markers.

Nuclear DNA contents and chromosome numbers in Gilliesieae

Here we report C-values for 27 species of Gilliesieae (Table 1), this study being the first attempt to survey GS evolution in the group. In general, flow cytometric profiles revealed high resolution histograms with 2C peaks for the target sample and the reference standard < 5% (CV% 2.23–4.75). At a given ploidy, variation in C-value from different accessions of the same species was low (< 2%), falling within the limits of intraspecific variation previously reported by Doležel & Bartoš (2005).

Overall, 1C-values in Gilliesieae varied 5.87-fold, ranging from 10.32 pg/1C in *I. uniflorum* ($2n = 2x = 12$) to 60.64 pg/1C in *Leucocoryne coquimbensis* F.Phil. ex Phil. ($2n = 4x = 18$). Note that we also found a polyploid sample of *Leucocoryne* cf. *narcissoides* Phil. ($2n = 6x = 26$, 1C = 79.32 pg), with an even larger genome, but we do not include this value in our analyses since it is most likely the result of an artificial hybrid cross from cultivation.

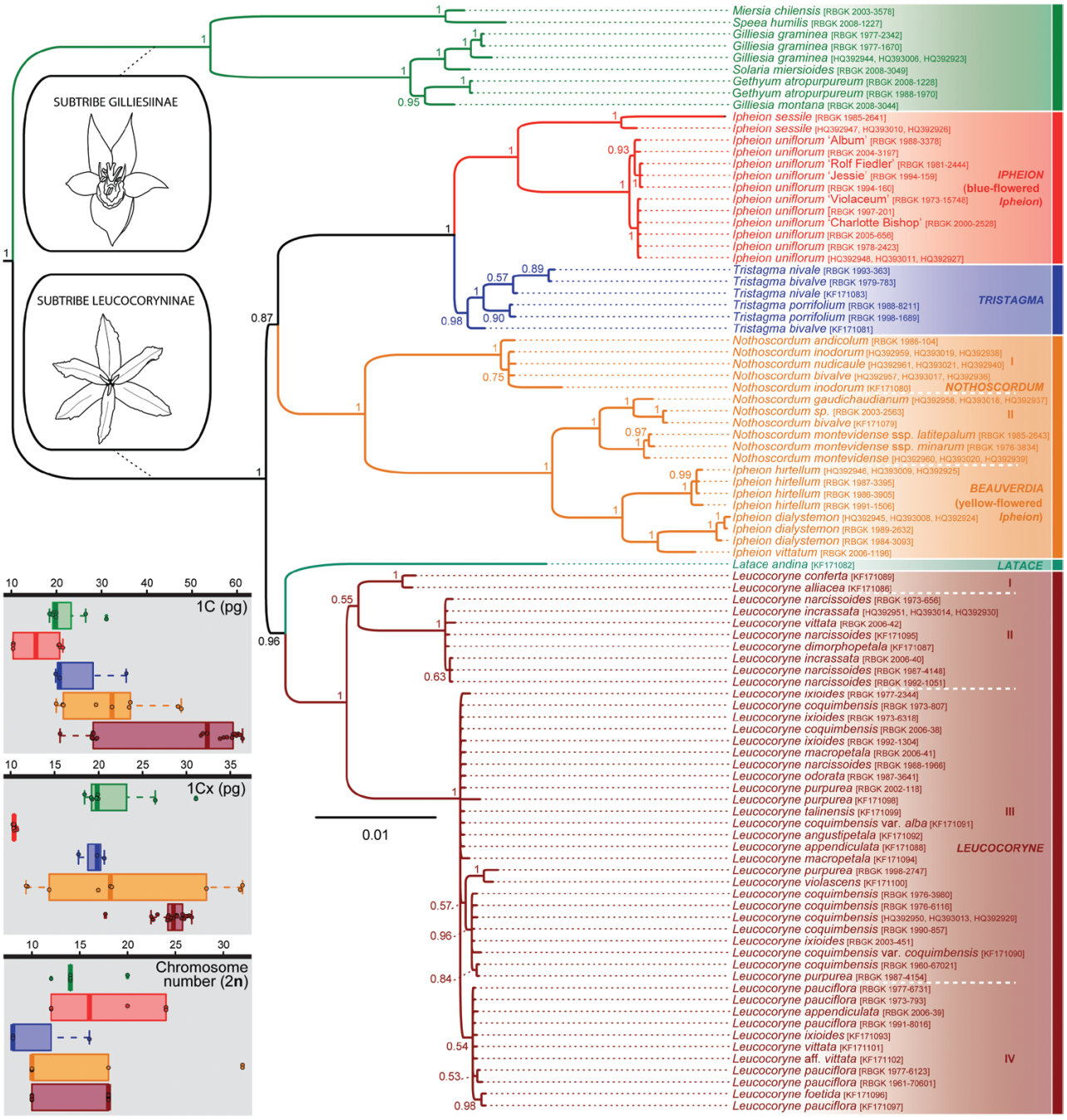

Figure 2. Majority-rule consensus phylogenetic tree of post-burn trees of Gilliesieae obtained through the Bayesian analysis of the ITS, *matK* and *ndhF* combined data set. Posterior probabilities (PPs) > 0.50 are indicated on nodes. Bottom left: boxplots with individual jitter values for accessions, depicting the distribution of 1C-values, 1C*x*-values and chromosome numbers in each of the clades of Gilliesieae.

At the monoploid (1C*x*)-level, *Nothoscordum s.l.* (including yellow-flowered *Ipheion*) was the most diverse among all genera studied (Table 1; Figs 2, 3). Monoploid (1C*x*) GS showed contrasting patterns among polyploid series. For example, we observed a relatively proportional GS increase across ascending ploidies in *Leucocoryne* (Fig. 3), supported by the relatively low variation of 1C*x*-values (1C*x* range 25.99–30.00 pg; Table 1), whereas genome downsizing was more apparent in polyploid *Nothoscordum* when compared to diploid species (1C*x* range 11.74–32.29 pg).

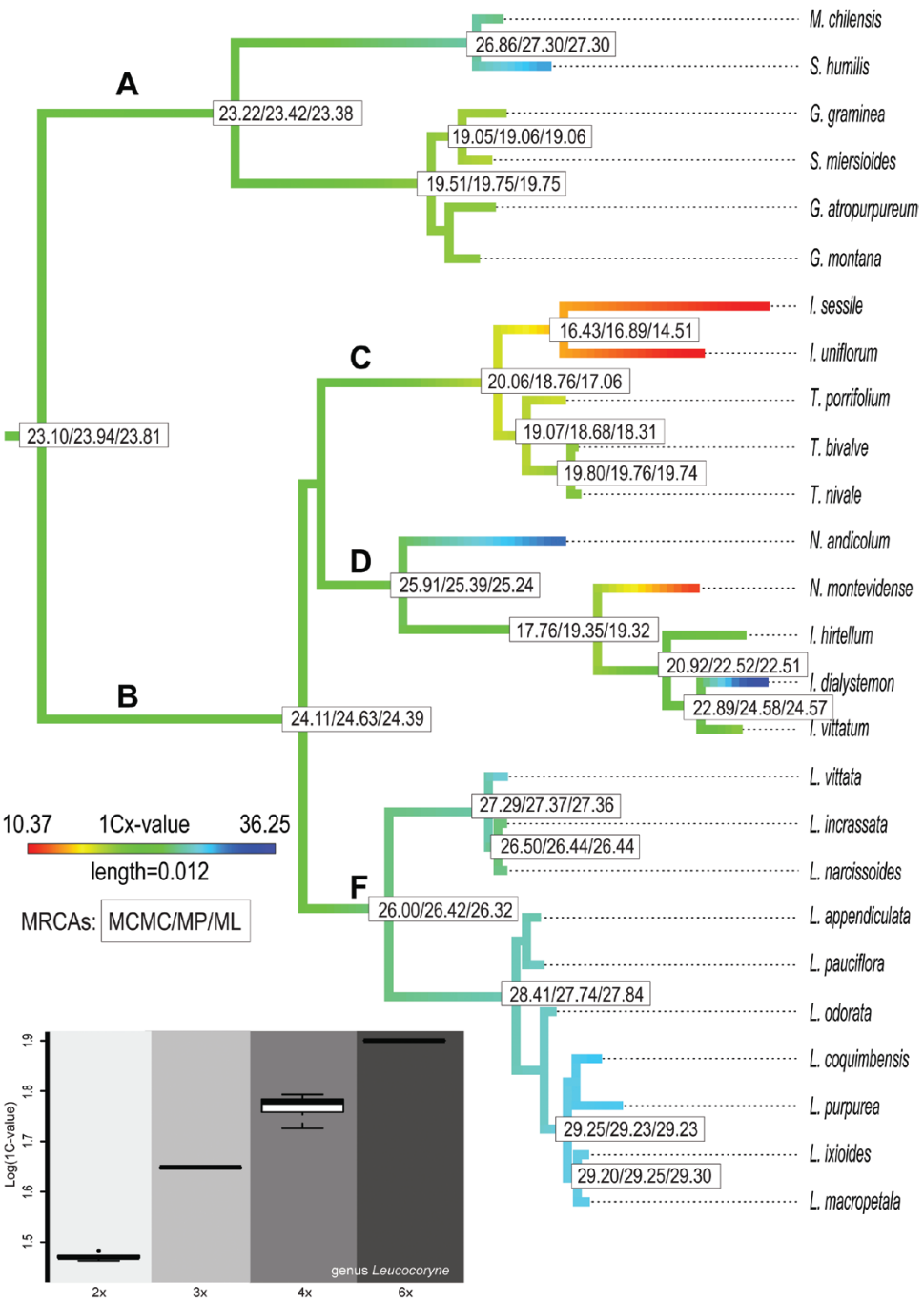

Figure 3. Ancestral genome size (1C*x*) reconstruction in Gilliesieae. Values in boxes represent the actual 1C*x*-values reconstructed using the different methods (i.e. Baysian inference [MCMC]/maximum likelihood [ML] and maximum parsimony [MP]) for supported nodes (PP > 0.95). Bottom left: Illustrative boxplots depicting the range of 1C*x*-values for each ploidy in *Leucocoryne*. PP, posterior probability.

Chromosome spreads were prepared de novo for most of the taxa studied (Table 1; Figure S1), although we also included previously published counts and karyotype data in further analyses (Figs 2, 4; Appendix S2). Chromosome numbers ranged from $2n = 8 = 6M + 2A$ [*Tristagma bivalve* (Hook. ex Lind.) Traub and *T. nivale* Poepp.; Fig. 4; Figures S1W, X] to $2n = 32 = 32M$ (*Nothoscordum montevidense* Beauverd; Fig. 4; Figures S1T, U) and ploidies included $2n = 2x$, $3x$, $4x$, $6x$ and $8x$ (Table 1; Fig. 4). A notable

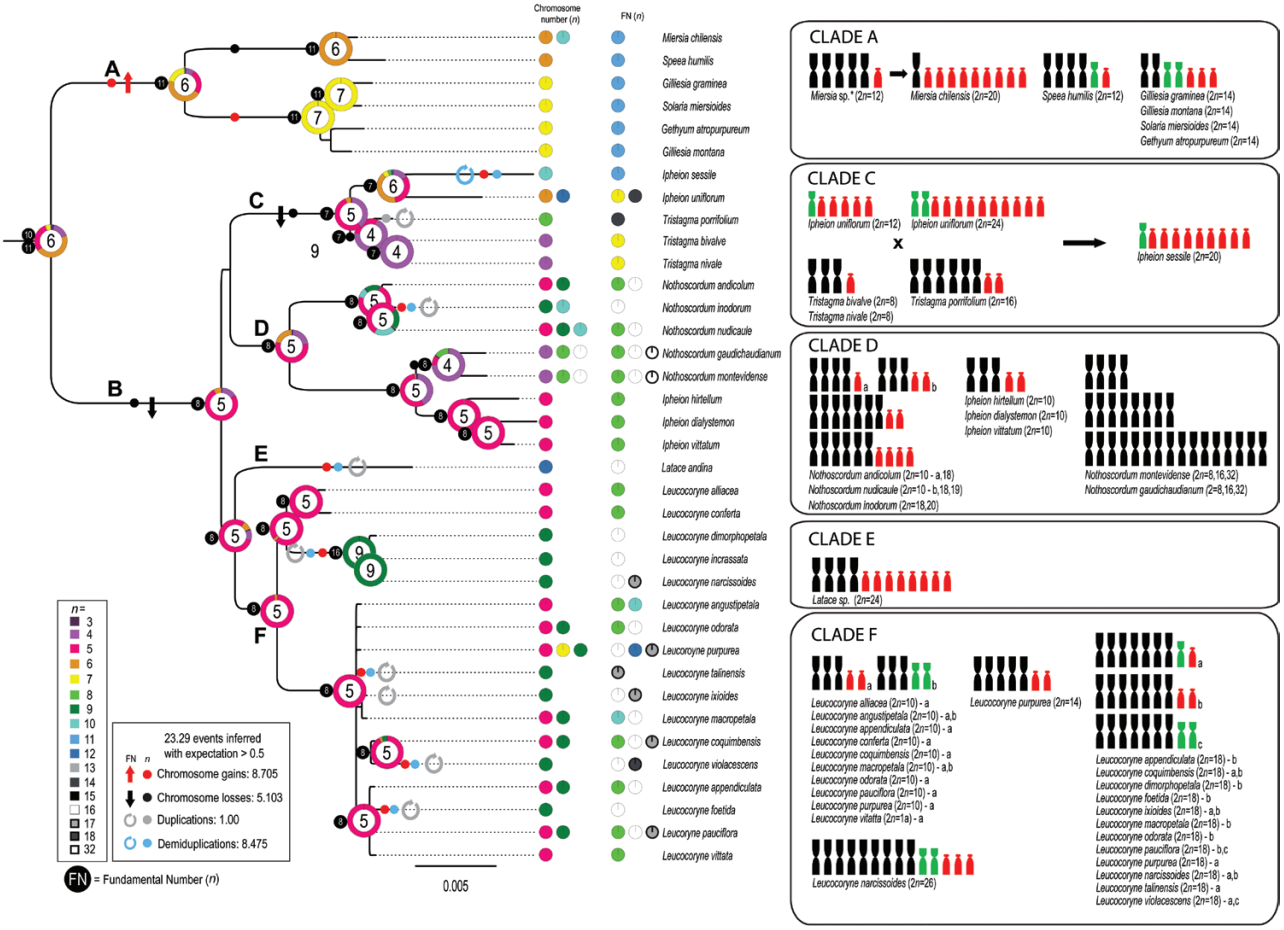

Figure 4. Chromosome and fundamental number (FN) reconstruction in Gilliesieae. Pie charts indicate haploid chromosome number and haploid FN. (i) Numbers inside the coloured pie charts at nodes represent the chromosome number with the highest probability. (ii) Numbers inside the black circles represent the FN reconstructed with the highest probability. As for the GS reconstruction, nodes lacking statistical support (PP < 0.95) were not considered for inferences. GS, genome size; PP, posterior probability.

range of karyotypes was recovered from the species studied (Fig. S1). The different karyotype formulae are detailed in Table 1 and, for illustrative purposes, schematic idiograms are depicted in Figure 4. Note that for a few species we could not determine their chromosome number; ploidy was therefore assigned on the basis of GS estimations.

Ancestral GS and chromosome reconstruction in Gilliesieae

The different methods used for GS reconstruction (MCMC, MP and ML) in Gilliesieae revealed relatively consistent inferred Anc1C*x*-values and only minor differences were observed for the MRCAs of selected nodes (Fig. 3). Indeed, each of the methods tested showed comparable patterns of GS evolution and the inferred values recovered for the deep nodes were highly congruent. For example, the MRCA of the tribe was reconstructed to have a 1C*x* = 23.10–23.94 pg (Fig. 3) under different methods.

Contrasting dynamics of GS evolution were found to be more prevalent in *Ipheion s.l.*, *Nothoscordum* and *Tristagma* (Fig. 3, clades C and D) than in *Leucocoryne* (clade F). We inferred an overall trend of GS downsizing across the lineage including *Tristagma* and blue-flowered *Ipheion* with respect to their MRCA (Fig. 3, clade C). However, among *Nothoscordum* and yellow-flowered *Ipheion*, such a pattern was lacking, and a more heterogeneous result was observed (Figs 2, 3, clade D). *Leucocoryne* was largely characterized by an overall trend towards GS increase during its diversification (Fig. 3, clade F). Under the Bayesian approach, the Bayes factor log likelihood scores supported the directional model, with contrasting GS ups and downs during the evolution of the tribe (Fig. 3). A lambda (λ)

score of 0.98 indicated the influence of phylogenetic relationships in contributing to the recovered GS variation, which probably took place gradually [kappa (κ) = 2.16].

Of the models tested for chromosome evolution in Gilliesieae, the four-parameter 'M3' (Glick & Mayrose, 2014) was the best fit for our data based on the lowest AIC values for FN and haploid (n) chromosome number. The results of the ancestral chromosome reconstructions using BI, their probabilities and the number of inferred chromosome events are depicted in Figure 4. Since the chromosome numbers and FN from BI and ML were consistent, only the results from BI are presented here. The MRCA of Gilliesieae was reconstructed to have a n = 6(5) and FN = 11(10) (Fig. 4), both haploid numbers and FN being reconstructed with relatively similar probabilities [i.e. n; p(5) = 0.46 and p(6) = 0.31]. Although n = 6 and FN = 11 were also recovered as the ancestral states for subtribe Gilliesiinae, in Leucocoryninae, n = 5 was recovered with the highest probability for the MRCA, coupled with a reduction of the FN from 11/10 to 8 (Fig. 4, clade B). Although the evolution of haploid chromosome numbers in Leucocoryninae was not clearly dominated by any specific type of event, chromosome reductions (i.e. fusions) were more frequent among the clade including *Ipheion*, *Nothoscordum* and *Tristagma* (i.e. clades C and D), whereas demiduplications coupled with chromosome gains (i.e. fissions) dominated in *Leucocoryne* (clade F). Polyploid events (whole genome duplication, WGD) tended to be reconstructed in derived positions along the tree [e.g. in *Ipheion sessile* Traub. and *Tristagma porrifolium* (Poepp.) Traub.]. The results from the FN reconstruction, recovered a simpler scenario, with only a further reduction of FN in the MRCA of *Tristagma* and blue-flowered *Ipheion*, and WGDs scattered across *Leucocoryne* (Fig. 4, clade F).

DISCUSSION

Generic delimitation and relationships in tribe Gilliesieae

This new phylogenetic hypothesis based on the analysis of three markers includes the most extensive taxonomic sampling so far used for reconstructing evolutionary relationships in Gilliesieae. As expected, it resolves Gilliesiinae and Leucocoryninae as two distinct, well-supported clades, in agreement with former taxonomic treatments based on morphological data (e.g. Rudall *et al.*, 2002). Indeed, early molecular inferences already recovered these two clades (although only two taxa of Gilliesiinae were included in the analysis; Fay & Chase, 1996; Fay *et al.*, 2006), as did the more recent analysis of Escobar (2012) based on an extended sampling focused on Gilliesiinae. Our efforts to compile, document and model karyological and cytogenetic traits across Gilliesieae enabled an evaluation of the phylogenetic signal of those characters at different taxonomic levels. Using these approaches, our results showed that GS values and chromosome numbers for Gilliesiinae fall within the overall ranges found in Leucocoryninae (see boxplots in Fig. 2), although each subtribe has its own set of distinctive karyotypes (Fig. 4).

Phylogenetic relationships in Gilliesiinae were largely congruent with Escobar (2012), revealing two main clades, one associating *Miersia* and *Speea* and the other including *Gilliesia* and related genera (Fig. 2). From the former, we only studied one species of *Miersia*, *M. chilensis*, but its karyotype, comprising nine acrocentric and one metacentric chromosome pairs ($2n$ = 20), contrasts strongly with those reported for other *Miersia* spp. studied, with $2n$ = 12, comprising one pair of acrocentric and five pairs of metacentric chromosomes, and which are similar to the karyotype of *Speea* (Escobar, Ruiz & Baeza, 2012; Fig. 4). In the clade of *Gilliesia* and relatives, all studied individuals of *Gethyum*, *Gilliesia* and *Solaria* showed the same karyotype and chromosome number ($2n$ = 14); these are found nowhere else in the tribe (Fig. 4). This clade, although being particularly well defined on the basis of molecular and karyological data, is also particularly problematic for generic delimitation. Certainly, (1) it combines topological inconsistencies between markers for statistically supported nodes and (2) *Gilliesia* is shown to be paraphyletic in its present circumscription (Escobar, 2012; Fig. 2). A detailed taxonomic revision is clearly needed, especially since this clade includes the type species of the tribe, *G. graminea* Lindl. The strong karyological homogeneity observed in *Gilliesia* and related genera could maybe help justify a broader generic delimitation. However, before making any taxonomic rearrangements, it will be necessary to gain a more comprehensive understanding of the evolutionary relationships in Gilliesiinae and this can only be achieved by obtaining phylogenetic data for *Ancrumia*, *Schickendantziella* and *Trichlora*, which have so far not been included in any molecular studies.

In Leucocoryninae, our phylogenetic inference recovered the three main clades previously described by Fay *et al.* (2006) and recently recovered by Souza *et al.* (2016): (1) a clade in which blue-flowered *Ipheion* are sister to *Tristagma*; (2) a clade containing a paraphyletic *Nothoscordum* and yellow-flowered *Ipheion*; and (3) the *Leucocoryne* clade sister to *Latace* (Fig. 2). As in former studies, efforts were made to expand the taxonomic sampling, but this failed to help in disentangling relationships among these main clades. Based on these data, it is clear that blue- and yellow-flowered *Ipheion* spp. do not form a natural group, a conclusion also

supported by the karyological evidence (Figs 2, 4). For example, *I. sessile* (probably a hybrid between *I. uniflorum* and a member of *Tristagma*) and *I. uniflorum* (a representative of blue-flowered *Ipheion*) have karyotypes comprising only submetacentric and acrocentric chromosomes (Fig. 4), even though *I. uniflorum* shares the same FN multiple of 14 as *Tristagma*, suggesting that one karyotype could have easily evolved from the other through a RT event. In contrast, *I. dialystemon* Guagl., *I. hirtellum* (Kunth) Traub and *I. vittatum* (Griseb.) Traub (representatives of yellow-flowered *Ipheion*) have one of the only two karyotypes that are not genus specific in Leucocoryninae (i.e. n = 3M + 2A); this is also found in some species of *Nothoscordum* and *Leucocoryne* (Fig. 4). Most representatives of the *Nothoscordum* clade have a FN of 16 (except in polyploid cytotypes, e.g. *N. andicolum* Kunth; Fig. 4), a trait supporting the cohesion of this group, which otherwise shows considerable cytological diversity comprising seven karyotypes and an impressive range in 1Cx-values (Figs 3, 4). The stability of FN suggests that once again, RTs are probably the main driver of karyotype evolution in the *Nothoscordum* clade.

In an attempt to resolve the paraphyletic status of *Ipheion* and after a detailed morphological analysis, Sassone, Giussani & Guaglianone (2014) suggested recognizing yellow-flowered *Ipheion* as a separate genus, for which they resurrected *Beauverdia*. However, in our molecular phylogenetic reconstruction *Beauverdia* is embedded in *Nothoscordum*, making the latter genus paraphyletic (Fig. 2), causing an additional problem rather than a solution. Since all yellow-flowered species already have combinations in *Nothoscordum*, the most nomenclaturally stable solution is to include the yellow-flowered *Ipheion* spp. in *Nothoscordum* and put *Beauverdia* back into synonymy.

The most recent molecular phylogenetic studies of Leucocoryninae by Jara-Arancio *et al.* (2014) and Souza *et al.* (2015) discuss the genus *Latace* [formerly *Zoellnerallium*; Crosa (1975)], a taxon with a convoluted nomenclatural history, based on *N. andinum* (Poepp.) Kunth ex Fuentes (= *Latace volkmannii* Phil.) and presenting a distinctive karyological profile (Souza *et al.*, 2015, 2016; Fig. 4). Our expanded phylogenetic reconstruction presented here places *Latace* as sister to *Leucocoryne* (PP = 0.96; Fig. 2), as in previous studies. The topology recovered for *Leucocoryne* was compatible with the four main *Leucocoryne* clades found by Souza *et al.* (2015), although with weaker resolution and support.

Genome dynamics in Gilliesieae

In contrast to the limited diversity in morphological characters, there is a wealth of cytogenetic and karyological traits that have probably played a key role in diversification of the group. As stated in the results, our analyses reconstructed n = 6(5)/FN = 11(10) as the ancestral haploid and fundamental chromosome numbers for the tribe and for the MRCA of Gilliesiinae (clade A, Fig. 4). With the exception of *M. chilensis* ($2n$ = 20), extant representatives of the subtribe have chromosome numbers of $2n$ = 12 and 14, supporting the split of the subtribe into two main clades. Based solely on chromosomal data, Goldblatt (1976) suggested that a reduction in chromosome number through RT (i.e. fusion) could have taken place, but more recently Escobar *et al.* (2012) suggested that an increase through chromosome fissions was more likely to have happened, taking into account that all species in the subtribe have the same FN. Our chromosome and FN modelling not only support this latter hypothesis, but also add further results suggesting that such chromosomal rearrangements did not have an impact on the FN, but were coupled with reductions in the overall GSs (clade A, Figs 3, 4). Changes in GS linked to RT events have been frequently reported, although the direction of GS change depends on the taxa analysed. For example, Enke, Fuchs & Gemeinholzer (2011) provided evidence of genome downsizing in *Crepis* L. coupled with descending dysploidy, x = 4 to x = 3, and accompanied by a reduction in total karyotype length. In contrast, Mas de Xaxars *et al.* (2016) found both genome upsizing and downsizing in dysploid species of *Artemisia* L. (x = 9 to x = 8), suggesting a differential impact of genomic restructuring after RT events.

As mentioned above, although most *Miersia* spp. studied to date have $2n$ = 12 (Escobar *et al.*, 2012), *M. chilensis* stands out as a dramatic example of chromosomal restructuring, which has led to an increase of chromosome number ($2n$ = 20; Fig. S1C). The fact that $2n$ = 12 and $2n$ = 20 have the same FN provides solid evidence that RT has played a major role in reshaping the karyotype of this species, resulting in a karyotype dominated by acrocentric chromosomes (Fig. 4). We were unable to obtain samples from further *Miersia* spp., preventing us from confirming if the observed GS reduction in this species compared with *Speea* is a direct consequence of such a chromosomal reorganization.

Although there is some uncertainty in the reconstructed haploid number for the MRCA of Gillisieae, our chromosome modelling provides some support for a reduction of chromosome number and FN in the MRCA of Leucocoryninae (from n = 6 to 5, FN = 10/11 to 8; Fig. 4). Although this suggests that there were early RT events predating the diversification of this subtribe, it is notable that these changes did not have a significant impact on the GS of the MRCA of the subtribe (Fig. 3). It is nonetheless worth mentioning that, although subsequent chromosome losses, WGDs and potentially, also hybridization (e.g. *I. sessile*), led to

changes in chromosome number in clades C (blue-flowered *Ipheion* and *Tristagma*) and D (*Nothoscordum* and yellow-flowered *Ipheion*), only a single decrease in FN was reconstructed for the MRCA of clade C (Fig. 4). Certainly, in the case of blue-flowered *Ipheion*, aside from recent WGDs, RTs have played a significant role in driving chromosome evolution, leading not only to karyotypes mainly dominated by acrocentric chromosomes, as already pointed out Souza, Crosa & Guerra (2010), but also to dramatic reductions in their monoploid GS (Fig. 3). Changes in karyotype symmetry have been reported to not only influence average chromosome sizes, but also result in GS changes (e.g. Hidalgo *et al.*, 2008; Peruzzi, Leitch & Caparelli, 2009; Chochai *et al.*, 2012). However, it is unclear how often such changes lead to genomic expansions or contractions. In the present study, the dominance of acrocentric chromosomes arising from RTs was accompanied by substantial genome downsizing in blue-flowered *Ipheion*, which is also illustrated by their relatively smaller overall haploid karyotype length compared with their relatives [i.e. *I. uniflorum* ($2n$ = 12, *c.* 53 μm), *I. sessile* ($2n$ = 20, *c.* 90 μm), *Tristagma bivalve* ($2n$ = 8, *c.* 90 μm), *Nothoscordum* spp. ($2n$ = 10, 60–70 μm), *Leucocoryne* spp. ($2n$ = 10, 90–150 μm)] (Souza *et al.*, 2010; Jara-Arancio *et al.*, 2012; Fig. S1). Conversely, in *Paphiopedilum* Pfitzer (Chochai *et al.*, 2012) an increase in telocentric chromosomes arising by centromeric fission was followed by GS expansions and in Liliaceae, Peruzzi *et al.* (2009) observed that increased karyotype asymmetry was also frequently accompanied by increases in GS. Considering these observations, it is clear that RTs are an important evolutionary mechanism that not only has an impact on chromosome number and structure but can also drive changes in GS by triggering amplification or elimination of DNA. In fact, besides polyploidy, RTs are probably the main mechanism driving the contrasting GS diversity found in *Nothoscordum* and yellow-flowered *Ipheion* (clade D, Figs 3, 4).

The analysis of chromosome evolution in *Leucocoryne* highlighted the presence of several, distinctive mechanistic processes inferred to be responsible for generating chromosome number changes in the group (Fig. 4), and which are likely to contribute to the lack of proportional chromosome number increase between diploids ($2n$ = 10) and polyploids ($2n$ = 18). In an attempt to deal with this issue by adding the FN reconstruction, the model supported the presence of WGDs in the MRCA of the polyploid lineages, instead of the demi-duplication combined with chromosome gains inferred solely from using the haploid chromosome numbers in the probabilistic modelling of chromosome evolution (Fig. 4, clade F). This new approach has brought novel insights into the probable evolutionary processes involved in chromosome number changes and highlights the utility of using FN instead of or as well as the haploid number to improve understanding of chromosome evolution in groups that have undergone recurrent chromosomal restructuring via RT. Besides that, our ancestral GS reconstruction revealed an initial GS expansion in the MRCA of the genus before the split into several clades, which was followed by subsequent increases (Fig. 3). Such results provide evidence for (1) a trend towards the accumulation of repetitive DNA and (2) little effect of WGD causing genome downsizing at the 1C*x*-level, which has been frequently reported in other polyploids (Leitch & Bennett, 2004). Setting polyploidy aside, the proliferation of repetitive DNA (including transposable elements and tandem repeats) is clearly a key driver of GS changes among species (Kejnovsky, Hawkins & Feschotte, 2012). For example, an extensive genomic analysis in Fabeae (Fabaceae) revealed that the differential accumulation of repetitive DNA accounted for 85% of the differences in GS among species (Macas *et al.*, 2015). In summary, it is certainly the balance between amplification and efficiency in eliminating extra DNA that controls overall GS. Delving deeper into the genomes of Gilliesieae to determine the types of DNA sequences involved in generating GS changes in the different clades will no doubt provide further insights into the role that transposable elements and other repetitive DNAs have played in generating the GS diversity encountered.

CONCLUSIONS

This study has compiled a comprehensive evolutionary framework for Gilliesieae, highlighting the importance of integrating cytogenetic and karyological information to understand better the evolution of this plant group in which recurrent chromosomal rearrangements have taken place. According to our results, RTs and polyploidy have not only been important for the diversification of the tribe, but can also be seen as triggers for generating the contrasting patterns of GS evolution uncovered in the group.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Mitotic chromosome numbers and associated karyotypes in Gilliesieae: (Gilliesiinae) A. *Gethyum atropurpureum* (2*n* = 14), B. *Gilliesia graminea* (2*n* = 14), C. *Miersia chilensis* (2*n* = 20), D. *Solaria miersioides* (2*n* = 14), E. *Speea humilis* (2*n* = 12). (Leucocoryninae) F. *Ipheion dialystemon* (2*n* = 10), G. *Ipheion hirtellum* (2*n* = 10), H. *Ipheion uniflorum* (2*n* = 12), I. *Ipheion uniflorum* (2*n* = 24), J. *Ipheion sessile* (2*n* = 20), K. *Ipheion vittatum* (2*n* = 10), L. *Leucocoryne coquimbensis* (2*n* = 10), M. *Leucocoryne coquimbensis* (2*n* = 18), N. *Leucocoryne ixioides* (2*n* = 18), O. *Leucocoryne* cf. *narcissoides* (2*n* = 26), P. *Leucocoryne narcissoides* (2*n* = 18), Q. *Leucocoryne pauciflora* (2*n* = 10), R. *Leucocoryne purpurea* (2*n* = 10), S. *Leucocoryne purpurea* (2*n* = 14), T. *Nothoscordum montevidense* subsp. *latitepalum* (2*n* = 32), U. *Nothoscordum montevidense* subsp. *minarum* (2*n* = 32), V. *Nothoscordum andicolum* (2*n* = 10), W. *Tristagma bivalve* (2*n* = 8), X. *Tristagma nivale* (2*n* = 8), Y. *Tristagma porrifolium* (2*n* = 16). Scale bar = 10 μm.

Appendix S1. Primers used for *ndhF* sequencing. Diagram showing (a) the placement of the original *ndhF* primers (Pires & Sytsma, 2002) and (b & c) the primers designed to obtain *ndhF* sequences for *Speea humilis* and *Miersia chilensis*, with indication of their sequences.

Appendix S2. Chromosome numbers previously reported and published.