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Genomic, karyological and morphological changes of South American garlics (*Ipheion*) provide insights into mechanisms of speciation in the Pampean region

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Abstract

Speciation proceeds through mechanisms that promote reproductive isolation and shape the extent of genetic variation in natural populations, and thus its study is essential to understand the evolutionary processes leading to increased biodiversity. Chromosomal rearrangements are known to facilitate reproductive isolation by hybrid sterility and favour speciation events. The genus *Ipheion* (Amaryllidaceae, Allioideae) is unique as its species exhibit a remarkable karyological variability but lack population-level genetic data. To unveil the diversification processes acting upon the formation of new lineages within *Ipheion* in the Pampas of South America, we combined morphology and karyology approaches with genotyping-by-sequencing. Our phylogenomic and population genomics results supported the taxonomic division of *Ipheion* into three morphological and genetically well-differentiated groups. The origin of *Ipheion uniflorum* was traced back to its current southern distribution area in the southern Pampean region (in Argentina), from where it had expanded to the north reaching Uruguay. Our results further suggested that chromosome rearrangements and ploidy shifts had triggered speciation events, first during the origin of *I. uniflorum* and later during its subsequent diversification into *I. recurvifolium* and *I. tweedieanum*, in both cases reinforced by extrinsic factors and biogeographical settings. The current study illustrates the analytical power of multidisciplinary approaches integrating phylo- and population genomics with classic analyses to reveal evolutionary processes in plants.

KEYWORDS
Amaryllidaceae, chromosome rearrangements, genotyping-by-sequencing (GBS), Robertsonian translocations, single-nucleotide polymorphisms (SNPs), spring starflower

1 | INTRODUCTION

The South American continent holds one of the greatest biodiversity hotspots on Earth. The emergence and maintenance of such biodiversity are complex and involve multilevel interactions of biotic and abiotic traits (Cantidio & Souza, 2019), and for most South American biomes and geographical regions, details on the underlying mechanisms are just starting to be revealed (e.g., Turchetto et al., 2014; Turchetto-Zolet et al., 2013). The Pampean region or Pampean phytogeographical province occurs between 30°S and 40°S in Uruguay,

central, eastern and northeastern Argentina, and southern Brazil (Cabrera, 1976 ; Soriano, 1991). This region comprises a floristic unit dominated by grasslands, with patches of steppes and savannas (Rivas Martínez et al., 2011). In this flat landscape, only the presence of broad rivers such as the Uruguay, Paraná and La Plata can represent geographical barriers to dispersal among plant populations (Turchetto et al., 2014) and, hence, it represents a remarkable natural environmental to study biogeographical processes. Two geographically distant mountain ranges, the Tandilia and Ventania (reaching 1,234 m a.s.l.), occur and hold high diversity and endemicity but have low impact on species' gene flow in the surrounding Pampas (Crisci et al., 2001). The study of grassland biodiversity in the Pampas and its components (such as *Ipheion* species, see below) is relevant to understand the processes responsible for the diversification of the native plant species and to evaluate *in situ* the impact of habitat fragmentation on genetic variability of species (Turchetto et al., 2014).

Information on genetic variation, its distribution within natural populations and association with the ecological context are fundamental to reveal how the formation of new lineages has occurred (Hewitt et al., 2001). In general, the process of speciation can be considered as a "speciation continuum" encompassing a process for gradual genetic, physiological and morphological changes that result in population divergence and in the emergence of reproductive isolation barriers (Shaw & Mullen, 2014). Changes in complete chromosomal sets (polyploidy) or individual chromosomes (chromosomal rearrangements) contribute to sympatric or parapatric speciation, by either pre- or postzygotic isolation (Levin, 2002; Moran et al., 2020; Rieseberg, 2001). The role of selection in the establishment and maintenance of chromosomal changes (particularly polyploidy) is known, but how underdominant chromosome rearrangements became fixed in natural populations is not yet fully understood (Marks, 1978; Rieseberg & Willis, 2007). Chromosomal rearrangements can facilitate reproductive isolation and even trigger incipient speciation (Lowry et al., 2008; Noor et al., 2001; Wang et al., 2020). For example, inversions (i.e., when a chromosomal segment reversed end to end) and translocations (i.e., a chromosomal segment is transferred to a new site in the same chromosome or a nonhomologous chromosome) are widely known to be associated with the suppression of meiotic and gametic recombination, a circumstance that is probably advantageous at the local scale for the initial establishment of a small, reproductively isolated, homozygous population (Feder et al., 2014; Fuller et al., 2018; Potter et al., 2017). In vascular plants, genomic studies are facilitating the identification of inversions and translocations in a variety of wild and domesticated plants (Huang & Rieseberg, 2020; Qin et al., 2014). Robertsonian translocations involve either the fusion of two telocentric or acrocentric chromosomes into one metacentric (or submetacentric) chromosome or the reverse process by centric fission. In general, Robertsonian translocations do not alter the number of chromosome arms, and in most cases, differences in the karyotype, morphology or genetic diversity are noticeable between the organisms with and without the translocation (Stebbins, 1950).

The tribe Leucocoryneae (Allioideae: Amaryllidaceae) includes six genera and ~150 species, all but one of these taxa occurring only in South America (Sassone et al., 2014). Within the subfamily Allioideae, the tribe originated ~37–31 million years ago (Sassone & Giussani, 2018) and they have diversified in terms of number and geographical range of species (Costa et al., 2020). Species of the tribe Leucocoryneae exhibit a variety of ecological adaptations (occurring in high elevations in the Andes to sea level in the Pampas prairies), of morphological traits (from uniflowered species to species having bi- or multiflowered inflorescences, showing a variety of flower pigmentation), and interesting karyological variability among genera. Studies using cytogenetic approaches (e.g., Crosa, 1972; Nuñez, 1990; Souza et al., 2010) have shown that chromosomal rearrangements and polyploidization played a role in the diversification of the tribe, mechanisms absent in sibling tribes (Escobar et al., 2020; Pellicer et al., 2017). Such cytogenetic mechanisms are known to facilitate speciation events and they might account for the tribal diversity. However, detailed population-level studies are missing and there is a lack of information regarding the genetic consequences of such structural chromosomal changes among plants. Recent studies (Sassone et al., 2018; Souza et al., 2010) have not only confirmed the above variation of ecological, morphological and karyological traits among genera within the Leucocoryneae, but have also revealed that the three species within the genus *Ipheion* Raf. displayed a remarkable number of karyotype changes derived from three different basic chromosome numbers (x = 5, 6, 7). Phylogenetic analyses using plastid and nuclear DNA markers showed *Ipheion* to be monophyletic and closely related to *Tristagma* Poepp. (Pellicer et al., 2017; Sassone & Giussani, 2018; Souza et al., 2016), a genus with 14 species but displaying stable karyotype morphology (Crosa, 1981). Since *Ipheion* species coexist, show little morphological variation and have a stable monoploid DNA content (Sassone et al., 2018), it seems likely that distinct chromosomal changes have supported speciation events. Such karyological diversity among *Ipheion* species is unique in the tribe and makes this genus a suitable model for investigating species diversification processes.

Ipheion is endemic to the South American Pampas with populations in Argentina's Buenos Aires and Entre Ríos provinces and, Uruguay, the origin for which dates to the Middle to Late Miocene ~17–10 million years ago (Sassone & Giussani, 2018). Three Pampean species are included in the current taxonomic definition of *Ipheion* (Sassone et al., 2014, but see Sassone et al., 2021): *I. recurvifolium* (C.H. Wright) Traub, *I. tweedieanum* (Griseb.) Traub and *I. uniflorum* (Graham) Raf. The last species has been cultivated in Europe since the beginning of the 19th century when the naturalist Tweedie sent bulbs to England (Sassone et al., 2017). Nowadays, *I. uniflorum*, the spring starflower, is an ornamental species cultivated worldwide. Different cultivars are characterized by a significant variation in flower colour (Castillo, 1986). *I. recurvifolium* (previously circumscribed as *I. sessile*, see Sassone et al., 2021) is also cultivated in Argentina and the UK, although is not as commonly used as ornamental.

Among natural populations a variety in flower colours, size and number of tepals has been detected. However, little is known about

their genetic variability or the distribution of trait-associated genotypes in the Pampean region. Hence, assessing the extant genetic variability present in natural populations of *I. recurvifolium*, *I. tweedieanum* and *I. uniflorum* would help unveil the mechanisms underlying species differentiation, the molecular consequences of chromosomal changes and their role in promoting local adaptation. Therefore, we used genotyping-by-sequencing (GBS) on *Ipheion* populations spanning the distribution areas of three species together with karyological and morphological analyses to disentangle their evolutionary histories, patterns and process of speciation within the genus. We aimed to (a) evaluate the morphological and karyological variability at the species and population levels within the distribution area of *Ipheion* species; (b) assess extant genetic variation, its geographical structure and admixture within and among populations and species of *Ipheion*; and (c) examine associations among genetic groups with morphological and karyological characters to reconstruct the evolutionary history of the genus. Putative factors responsible for the karyotypic evolution and driving speciation within *Ipheion* are discussed.

2 | MATERIALS AND METHODS

2.1 | Plant materials

Field trips were conducted to the native area of the three species of *Ipheion* in southern Uruguay and the Argentinian provinces of Buenos Aires and Entre Ríos. A total of 88 individuals were sampled across 20 natural populations (Table S1), and three outgroups were included for phylogenetic analyses: *Beauverdia vittata* (Griseb.) Herter (Giussani, L. 429, Sassone, A. 83) and *Tristagma sessile* (Phil.) Traub (Arroyo, M.T.K. 29124). Selected individuals of each population were kept alive and cultivated in a glasshouse at the Darwinion Institute of Botany (Buenos Aires, Argentina) and the Albrecht-von-Haller Institute for Plant Sciences (Göttingen, Germany). Voucher specimens from each collection site were stored at SI.

2.2 | Morphometric analyses

Based on a previous study (Sassone et al., 2013), our morphological exploration was limited to reproductive characters. In total, 55 specimens bearing flowers were studied, representing 56% of the total samples used to produce the genomic data set. We measured 16 quantitative and four qualitative traits (Table S2).

To assess the morphological similarity among specimens and populations, principal coordinate analysis (PCoA) was performed using the R package "ape" (Paradis & Schliep, 2019) and Gower's dissimilarity coefficient, a measure of choice for mixed numeric/categorical data (Gower & Legendre, 1986), was used with the R package "FD" (Laliberté & Legendre, 2010). Eigenvalues were used to project the relative magnitude of morphological vectors among specimens dispersed in two-dimensional space. A principal component analysis (PCA) based only on quantitative traits was also calculated using the basic R function "prcomp." Missing values were replaced by the average value per character per species. Pearson's coefficient (r) was used to estimate the correlation between pairs of characters, which were then clustered using a hierarchical clustering order and plotted in a correlogram using the "corrplot" R package (Wei & Simko, 2017). Finally, we used 10 quantitative noncorrelated characters in a discriminant analysis (DA) to indicate the goodness of classifications using the a priori groupings (species). Discrimination accuracy was evaluated using a leave-one-out cross-validation procedure, and the percentage of each individual correctly assigned to one of the three *Ipheion* species was computed. A biplot of the two linear discriminant axes was performed with the R package "ggord" (Beck, 2017).

2.3 | Karyological analyses

Root tips were collected from bulbs and pretreated with 0.05% colchicine for 6 hr at room temperature, fixed in ethanol/acetic acid (3:1, v/v) for 12–48 hr and stored in 70% ethanol at 4°C. Fixed root tips were hydrolysed in 1M HCl for 10 min at 60°C, and stained for at least 20 min using the Feulgen reaction (Sigma Aldrich, 1090341000). Individual meristems were macerated in a drop of 2% acetic orcein, squashed and analysed with a Leica DM5500B microscope (Leica Microsystems). For each population, between three and six metaphases with clear chromosome morphology were selected and chromosome measurements were done using the Leica APPLICATION SUIT software (version 4.1.0). Images were acquired with a DC450 camera (Leica Microsystems). Chromosomes were classified using the arm ratio (r = length of the long arm/length of the short arm) as metacentric (r = 1.00–1.49), submetacentric (r = 1.50–2.99), acrocentric ($r \geq 3.00$) or telocentric ($r = \infty$) following Guerra (1986)

Mean lengths of the whole chromosome complement and karyotype symmetries were used to reveal possible karyological differentiation among populations and species. Interchromosomal asymmetries were estimated using the A_2 index proposed by Romero Zarco (1986). Intrachromosomal asymmetries were estimated using four different indexes: the total form percentage (TF%; Huziwara, 1962), the karyotype asymmetry index percentage (Ask%; Arano, 1963), the symmetric index (Syi; Greilhuber & Speta, 1976) and the intrachromosomal asymmetry index A_1 (Romero Zarco, 1986). The four categories of Stebbins (including three subtypes on each category; Stebbins, 1971) were also used to characterize intrachromosomal asymmetries. The variation between the mean values of each asymmetry index among populations was compared statistically by ANOVA followed by Tukey's test.

2.4 | Genomic analysis

2.4.1 | DNA extraction and GBS

For each sample, DNA was extracted from leaf tissue preserved in silica gel. DNA extraction was carried out following a modified CTAB protocol as in Sassone and Giussani (2018). For library

preparation, 20 ng of genomic DNA was used and cut with two restriction enzymes, *Pst*I-HF (NEB) and *Msp*I (NEB). Individual barcoding and single-end sequencing on the Illumina HiSeq 2000 followed Wendler et al. (2014). GBS library construction and sequencing were performed at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Germany. Quality assessment of all raw sequence samples was performed using FASTQC (Stamatakis et al., 2005).

2.4.2 | Data assembly, data exploration

GBS loci were assembled using the IPYRAD version 0.9.56 pipeline (Eaton & Overcast, 2020). The running parameters were set using default recommendations (available from the IPYRAD documentation) and exploring different settings after Eaton et al. (2017) and Gargiulo et al. (2021). Briefly, assemblies were generated employing clustering thresholds c = 0.85 and a minimum number of four samples per locus. Statistical base calling was conducted with a maximum of five Ns in consensus sequences. To explore single nucleotide polymorphism (SNP) data, a PCA was performed using the IPYRAD API toolkit. The potential effect of linkage between SNPs was reduced by subsampling one SNP per locus.

2.4.3 | Phylogenomic analyses

Phylogenetic relationships were inferred by a maximum-likelihood analysis using the GTR+Γ substitution model and a data set with 32 ingroup samples of *Ipheion* (representing different populations of the three species) plus the three outgroups (data set 1, Table 1). The tree search was conducted through an estimation of the proportion of invariable sites, and a total of 100 nonparametric bootstrap replicates performed in RAXML 8.2.10 (Stamatakis, 2014) as implemented in the IPYRAD API analysis tool (https://ipyrad.readthedocs.io/en/latest/API-analysis/). Results were summarized using a 50% majority-rule consensus tree. The phylogenetic relationships among species were also inferred by a coalescent-based method, the SVDQuartets algorithm (Chifman & Kubatko, 2014), implemented in TETRAD version 0.9.13 (http://github.com/dereneaton/ipyrad). We inferred all possible quartet trees based on a matrix of one randomly selected SNP per locus (quartet sampler [random, nsamples**2.8]: 22784/58905). One thousand nonparametric bootstrap replicates were conducted and the results were summarized into a 50% majority-rule consensus tree plotted in the R environment with the packages "ape" and "ggplot2." To consider reticulate evolution and explore incongruence among loci, DNA matrices were analysed with a Neighbor-net network approach derived from uncorrected P-distances using SPLITSTREE5 version 5.1.4-beta (Huson & Bryant, 2006).

2.4.4 | Population genomics

The Bayesian clustering method STRUCTURE version 2.2.4 (Pickrell & Pritchard, 2012) was used to determine the number of distinct genetic clusters (K) with a burn-in period of 500,000 repetitions followed by 2,000,000 repetitions, as implemented in the IPYRAD API. Ten (data set 2) to 15 replicate analyses (data sets 3 and 4) were performed for each data set with values of K = 1–10. Replicates were summarized and visualized using the "pophelper" library (Francis, 2017). The STRUCTURE approach assumes that markers are not linked and that populations are panmictic (Pritchard et al., 2000). Then, to check results without this assumption we also perform a discriminant analysis of principal components (DAPC; Jombart et al., 2010). DAPC consists of performing a PCA to summarize genotypic variation among individuals and then a DA is performed to categorize the PCA results, maximizing the variation among a predefined set of groups and minimizing variation within them. The analyses were performed using the R package "adegenet." We conducted a cross-validation test with 1,000 permutations.

Population genetic summary statistics (H_O, H_E, H_T, F_{ST} and F_{IS}) were calculated to describe and compare overall and population-specific genetic diversity for data sets 2, 3 and 4 (Table 1), using the R package "hierfstat" (Goudet, 2005) and file conversions were facilitated with the R package "dartR" (Unmack et al., 2018). We also used the R function stamppFst from the R package "StAMPP" 1.6.2 (Pembleton et al., 2013) with 95% confidence interval (CI) estimated on 1,000 bootstraps to calculate the pairwise genetic distance (F_{ST}) for each of the subpopulations according to Weir and Cockerham (1984). For these analyses loci with two alleles were considered.

2.4.5 | Ploidy estimations

Genome-wide heterozygosity can be used for identifying ploidy variation. Here, the R package "gbs2ploidy" (Gompert & Mock, 2017) was used to estimate ploidy within species. This package infers cytotypes based on the allelic ratios of heterozygous SNPs identified during variant calling within each individual. Input data were

TABLE 1 Next generation sequencing characteristics of the four assembled genotyping-by-sequencing data sets under IPYRAD version 0.9.56

Data set	No. of specimens	No. of loci	Concatenated length (bp)	% Missing data
1 = OG+subsampling *Ipheion*	32	12,111	1,095,978	65
2 = *Ipheion*	84	26,456	2,389,167	75.8
3 = *Ipheion uniflorum* (natural populations)	56	19,507	205,766	66.3
4 = *Ipheion recurvifolium*	23	7,378	649,701	61.07

prepared using the vcf2hetAlleleDepth.py script (https://github.com/carol-rowe666/vcf2hetAlleleDepth) and results were corroborated by comparing samples with a priori unknown ploidy to samples with known ploidy.

All figures were prepared for publication using INKSCAPE version 0.92 (https://inkscape.org/).

3 | RESULTS

3.1 | Morphological differentiation

The ordination analyses (PCoA and PCA) showed similar results revealing that the three species of *Ipheion* are morphologically distinct units (Figure 1a; Figure S1a,b). The first two coordinates of the PCoA explained 65.8% of the total variation. *Ipheion recurvifolium* was differentiated from the other two species within the morphospace along Axis 1 due to the longer style and tepal tube (Figure 1a; Figure S1a,b). Meanwhile, *Ipheion uniflorum* was segregated from *Ipheion tweedieanum* mostly along Axis 2 (Figure 1a; Figure S1a). We checked the correlation among quantitative characters and constructed a correlogram to visualize them (Figure S1c). The characters of the gynoecium were strongly correlated to each other and with bract and pedicel length, and the tepal characters were strongly correlated among them ($r > 0.5$, Figure S1c). In the DA (performed only with quantitative noncorrelated characters), the a priori groups were markedly different (Figure S1d), and 100% of specimens were correctly grouped to the original species in cross-validated cases. *I. recurvifolium* can be discriminated based on style and tepal length, whereas *I. uniflorum* and *I. tweedieanum* can be differentiated by the length of most of the floral characters (*I. tweedieanum* has essentially smaller flowers; Figure S1b,d, Table S2). When observing morphological similarity within species, no population grouping was obtained (Figure S1a). Within *I. uniflorum* different flower colours are found only in single individuals within populations and no general pattern could be recognized per population.

3.2 | Karyotype variability

The karyotype formula of the three *Ipheion* species was corroborated. *I. recurvifolium* has 1 submetacentric (SM) +4 acrocentric (A) chromosomes ($2n = 4x = 20$); *I. tweedieanum* has 7 (A) chromosomes ($2n = 2x = 14$) and *I. uniflorum* has 1 submetacentric (SM) +5 (A) chromosomes ($2n = 2x = 12$) (Table S3). The karyotypes of six *I. uniflorum* populations covering most of the species geographical range were compared. All individuals and populations evaluated were diploids. The population 555_TA (Tandil) possessed the smallest chromosome (c min, Table 2) whereas one specimen from Bahia Blanca (BB) had the largest chromosome (c max, Table 2). The total sum of haploid chromosome length (TCL) for each population ranged from 108.9 µm in population 513_AZ (Azul) to 126.8 µm in BB (Table 2). For the interchromosomal asymmetry (A_2), the karyotype of population S51 (San Cayetano, SCa) was the most symmetric one ($A_2 = 0.03$), whereas the most asymmetrical karyotype was found in population 555_TA ($A_2 = 0.11$) (Table 2). However, the statistical test revealed no significant differences among populations ($F = 1.756$, $p = .203$). Similar results were obtained from the different intrachromosomal asymmetry indexes (see Table 2). The total form per cent (TF%) ranged from 8.63 in population 513_AZ to 9.45 in population 553_TA. The karyotype asymmetry index percentage (Ask%) is complementary to TF% and therefore showed a range between 90.55 in population 553_TA and 91.37 in population 513_AZ. The symmetric index (Syi) also showed little variation among populations, and the intrachromosomal asymmetry index A_1 was the same in all populations ($A_1 = 0.99$). All karyotypes fall within the category 1C of Stebbins, all having (8/12 = 0.67) acrocentric and/or telocentric chromosomes and ratios between the large and small chromosome arms <2.

The prediction of ploidy for each sample of *Ipheion* using "gbs2ploidy" was highly accurate as the inferred cytotypes matched in all cases the ploidy of confirmed samples by chromosome counts and/or by using flow cytometry and DNA content analyses (Table S3). All specimens of *I. tweedieanum* were inferred to be diploids, and all specimens of *I. recurvifolium* were inferred to be tetraploids. With regard to *I. uniflorum*, 87.5% of the samples ($n = 49$) were diploids and seven specimens were tetraploids (Table S3).

3.3 | Genomic analysis

3.3.1 | Sequencing, alignment and SNP calling

Investigation of all samples with FASTQC showed that raw reads were generally of good quality. After the initial filtering, the number of reads was reduced to an average of 1.05 million per sample. The characteristics of assembled GBS data sets generated with IPYRAD and used in phylogenetic and SNP-based analyses are summarized in Table 1.

3.3.2 | Genetic variability among *Ipheion* species

PCoA of a subsampling of one SNP per locus, with the two first components explaining 42.2% of the observed variance, showed that the three *Ipheion* species were differentiated (Figure 1e). Uncorrected P-distance-based split networks of the SNP matrix recovered species groups and displayed a partially reticulated pattern, whereby reticulation is almost exclusively found among populations within species but not among species, suggesting single origins of individual lineages (Figure S2a). *I. recurvifolium* and *I. tweedieanum* were sister species whereas *I. uniflorum* was more distant (Figure S2a). The genus and species clustering was highly supported when performing phylogenetic reconstruction using maximum likelihood (Figure 2b) and a coalescent-based method (Figure S2b), reflecting a similar topology regarding species relationships. Within *I. uniflorum* the southern

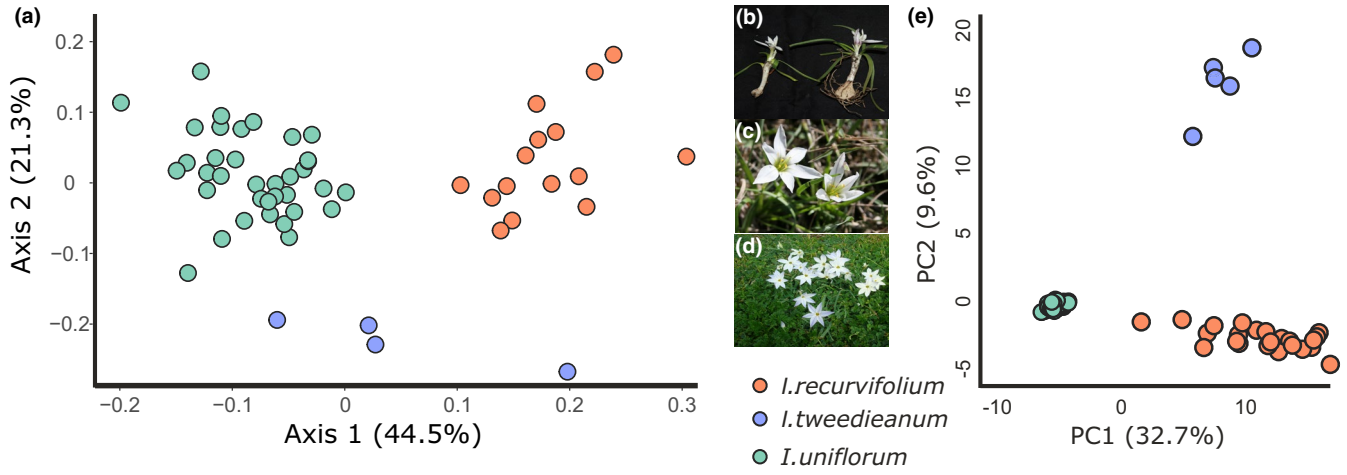

FIGURE 1 (a) Tridimensional plot of the first three principal coordinates (PCoA) showing the distribution of 55 operational taxonomic units based on 16 morphological characters (see Table S2). Photographs: (b) *Ipheion recurvifolium*, *Giussani et al. 487*; (c) *Ipheion tweedieanum*, *Giussani & Morrone 420*; (d) *Ipheion uniflorum*, *Giussani et al. 513*. Photo credits: Giussani, L. and Sassone, A. (e) First and second principal components for 84 *Ipheion* accessions based on 1,240/9,953 unlinked SNPs

TABLE 2 Intraspecific karyotype variability among *Ipheion uniflorum* populations

	BB	555_TA	553_TA	513_AZ	540_AZ	SCa	Tuckey's test
2*n*	12	12	12	12	12	12	–
X	6	6	6	6	6	6	–
TCL	126.8 ± 13.2	111.7 ± 12.1	111.2 ± 10.6	108.9 ± 8.1	114.9 ± 8.7	109.4 ± 2.5	–
C	10.6 ± 1.1	9.3 ± 1.0	9.3 ± 0.9	9.1 ± 0.7	9.6 ± 0.7	9.1 ± 0.2	–
c max	12.2	11.1	11.6	10.6	11.1	10.4	–
c min	7.9	6.7	6.9	7.0	7.7	8.2	–
i[a]	24.2	23.5	25.0	24.1	23.6	26.4	–
TF%	8.99	8.93	9.45	8.63	8.75	9.21	NS
Ask%	91.01	91.07	90.55	91.37	91.25	90.79	NS
Syi	9.88	9.80	10.43	9.45	9.58	10.15	NS
A1	0.99	0.99	0.99	0.99	0.99	0.99	NS
A2	0.10[A]	0.11 [A]	0.10 [A]	0.08 [A]	0.08 [A]	0.03[B]	S
r[a]	4.69	4.19	3.58	4.1	4.07	3.11	–
r>2	2	3	3	2	3	4	–
R	4.9	3.5	3.1	3.6	3.4	2.3	–
S cat	1C	1C	1C	1C	1C	1C	–

A1, A2 = intrachromosomal and interchromosomal asymmetry indices, respectively; c = mean chromosome length (μm); c max, c min = maximum and minimum chromosome length (μm); i = mean centromeric index; *r* = mean chromosome ratio (long/short arms) *r* > 2 = proportion of chromosome pairs with arm ratio >2; R = largest/smallest chromosome ratio; TCL = haploid complement length (μm). TF% = ratio between the total sum of short arms (*p*) and the total length of a chromosome set ×100; Ask% = ratio between the total sum of long arms (*q*) and the total length of a chromosome set ×100; Syi = ratio between the mean length of the short arms (*p*) and the mean length of the long arms (*q*) ×100. NS = not significant; S = significant at ≥0.05.

[a]Values based on four chromosome pairs with short arms.

populations plus one specimen from Saladillo (SA) were grouped with high support (BB, SCa, MBu; Figure 2a,b).

In the analysis of the SNP-based population structure for the three *Ipheion* species (data set 2, Table 1), Evanno's Δ*K* method suggested *K* = 2 and the next best fit at *K* = 3 and *K* = 6, identifying compositional differences among species (Figure 2c; Figure S3a,c). In population structure analysis with *K* = 2, *I. tweedieanum* and *I. recurvifolium* were clustered in the same group and specimens of *I. tweedieanum* presented 10–20% of admixture with *I. uniflorum*. Similar results were obtained when performing DAPC without the assumption of panmixia (Figure S4a,b).

The summary statistics for the three *Ipheion* species were concordant with other analyses. Despite its wide geographical range

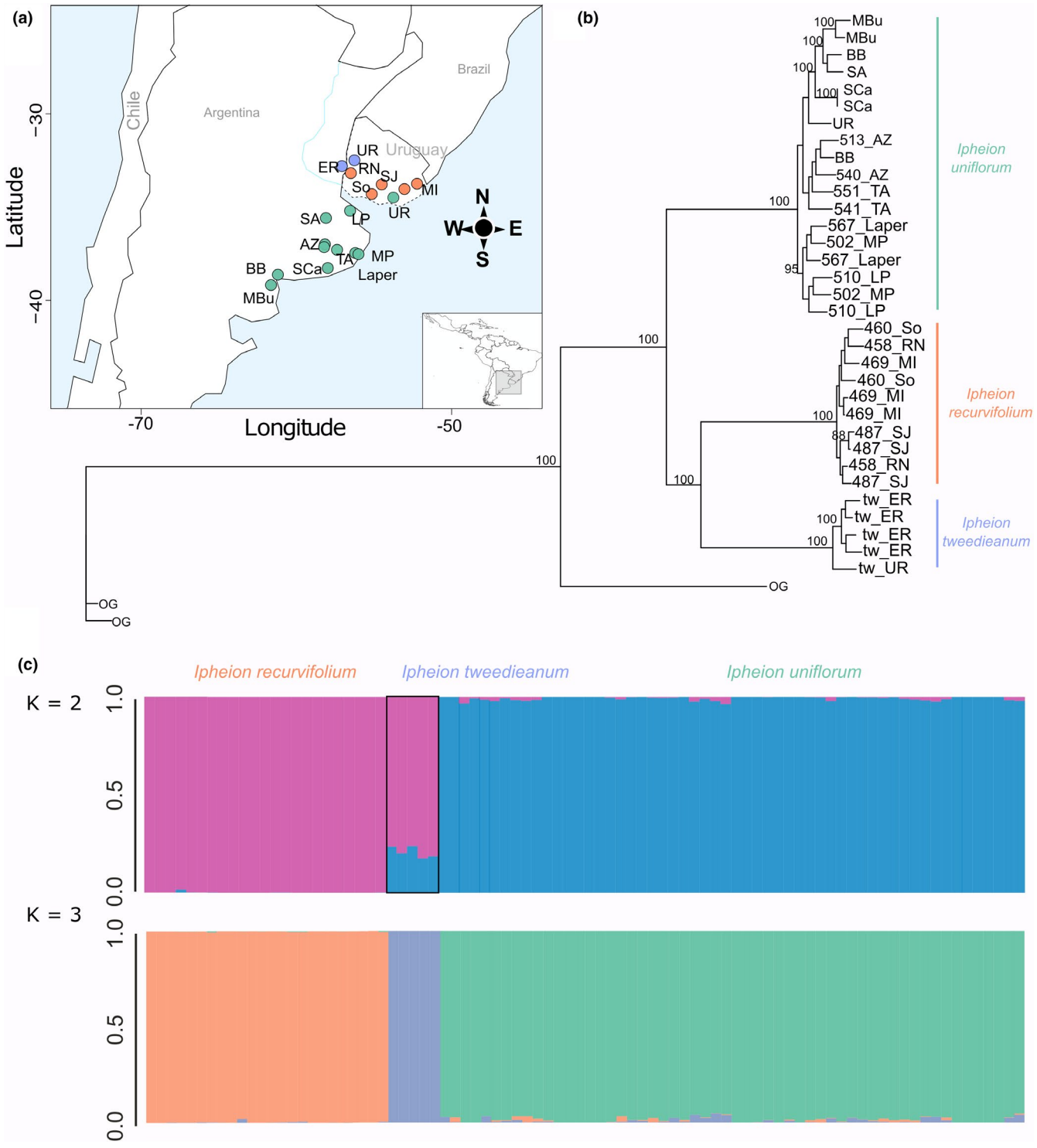

FIGURE 2 (a) Map showing sampling sites for *Ipheion* (coloured points indicate species). (b) Maximum-likelihood inference for 32 samples, representing sampled populations and three outgroups. Bootstrap values are indicated above branches. (c) Population structure analysis with K = 2 and 3 based on 6,409 unlinked SNPs of 84 specimens. Individuals are represented by a vertical bar that is divided by coloured segments representing the likelihood of membership to each cluster

and genetic variability, *I. uniflorum* showed a lower heterozygosity than expected (H_O =0.084, H_E =0.178) and the values observed for the other two species with more restricted distribution (H_O =0.172 for *I. recurvifolium*, and H_O =0.243 for *I. tweedieanum*). *I. uniflorum* also showed a higher inbreeding coefficient (F_{IS} =0.527) and fixation index (F_{ST} =0.334) than those observed for *I. recurvifolium* (F_{IS} =0.309, F_{ST} =0.122) or *I. tweedieanum* (F_{IS} =0.251, F_{ST} =0.086), suggesting restricted gene flow among populations along its distribution. However, the overall levels of genetic diversity were not different between *I. uniflorum* (H_T =0.267), *I. recurvifolium* (H_T =0.283)

or *I. tweedieanum* (H_T =0.354). Estimates of pairwise fixation indices between species pairs revealed similar values among species, and higher values for *I. uniflorum*: F_{ST} =0.733 (0.727–0.739) for *I. uniflorum–I. tweedieanum*, F_{ST} =0.574 (0.571–0.579) for *I. uniflorum–I. recurvifolium*, and F_{ST} =0.698 (0.693–0.703) for the pair *I. recurvifolium–I. tweedieanum*, indicating clear differentiation and reproductive isolation among species.

In the analysis of the *I. recurvifolium* populations (data set 4), Evanno's Δ*K* method determined the best-fit population models at *K* = 4 (Figure S5). All specimens showed various levels of admixture (15%–50%), potentially reflecting their tetraploidy, and no geographical correlation was recovered. DAPC suggested a similar grouping (Figure S4c,d).

3.3.3 | Genetic variability within *I. uniflorum*

Since exhaustive sampling was available for *I. uniflorum*, a new data set was produced including 10 localities (13 natural populations; Table S1) of this species (data set 3, Table 1) and analysed. SNP-based genetic structure analysis of *I. uniflorum* populations using Evanno's Δ*K* method determined the best-fit population models at *K* = 2 and 3 (Figure 3; Figure S3b,c). In both analyses one cluster grouped most of the specimens from the southern populations (BB, MBu, SCa) together with two specimens from one of the central populations (SA). The northern localities (UR and LP) were all grouped with the central populations (MP, LaPer, SA, AZ, TA). In all cases, the specimens showed a low to high degree of admixture (5%–50%). Similar groups were obtained when analysing DAPC results (Figure S4e,f). The summary statistics resulted in values of F_{ST} =0.37 and F_{IS} =0.53. Estimates of pairwise fixation indices between regions (Table 3) revealed the strongest differentiation for southern populations (BB, MBu, SCa; Figure 2a). The central population and northern populations showed lower differentiation.

4 | DISCUSSION

4.1 | Species variability in *Ipheion*

The small genus *Ipheion* includes three species (Figure 2b) that are differentiated by floral (Figure 1; Figure S1), genetic (Figure 2; Figure S2) and karyological traits. Phenological data (Table S4) indicated a shift in flowering time among *Ipheion* species. *Ipheion uniflorum* blooms in late winter (late August to the beginning of September) whereas *Ipheion recurvifolium* and *Ipheion tweedieanum* flower in autumn (May–June). Since flower morphology is more similar between *I. uniflorum* and *I. tweedieanum* (Figure 1) and observed changes in flower morphology were subtle except for the smaller size of the latter (Figure S1; and see Figure 1b–d), these species probably share similar pollinators. In the case of *I. tweedieanum* and *I. recurvifolium*, the species show no clear phenological shift, but *I. tweedieanum* occurs in a small geographical area on the eastern border of the distribution of *I. recurvifolium*, where it is adapted to flooding patches of the Uruguay River basin (Crosa & Marchesi, 2002). Moreover, in addition to the habitat differences, the flowers of *I. recurvifolium* bear a tube and style significantly longer than the other two species (Figure S1a,c; Table S2), suggesting this species is pollinated by different insects, probably Sphingidae (Lepidoptera; Sassone et al., 2021). Shifts in phenology and/or habitat between species would maximize individual fitness by increasing floral visitation, which can explain the lack of interspecific hybrids in nature (Sassone & Giussani, personal observation).

*I*pheion *uniflorum* has the widest geographical distribution, being found not only in plain grasslands but also in hills (e.g., in the Tandilia systems). This correlates well with the observed genetic variability and associated karyotype stability (1 SM +5 A chromosomes; see discussion in the next section), all consistent with a well-delineated species. Surprisingly, besides the widespread diploid individuals (2*n* = 2*x* = 12), we also detected single tetraploid individuals mixed among diploid *I. uniflorum* populations (2*n* = 4*x* = 24) through GBS heterozygosity estimations, which still require confirmation using chromosome counts. Up to now, tetraploid individuals have only been recorded in Uruguay (Souza et al., 2010). *I. recurvifolium* is restricted to Uruguay and southern Brazil and shares the habitat in both sympatry and parapatry with *I. uniflorum*. Individuals of *I. recurvifolium* were grouped separately with low genetic admixture of *I. tweedieanum* and no admixture from *I. uniflorum*. This was consistent with all individuals being tetraploids (2*n* = 4*x* = 20) with a unique karyotype (1 SM +4 A chromosomes). In polyploid plants, the occurrence of multiple genome sets foster genetic divergence through extra gene copies and increase genetic sequence variability (Otto, 2007; Soltis et al., 2015). *I. tweedieanum* also formed a consistent genetically differentiated group with moderate admixture from *I. uniflorum* and *I. recurvifolium* and a distinctive karyotype (7 A chromosomes, 2*n* = 2*x* = 14; see discussion in the next section).

The above changes in flower traits, phenology and habitat adaptation certainly contributed to reproductive isolation responsible for the formation of the three *Ipheion* species found in nature. In long-lived plants, even low levels of fertility of interspecific hybrids—including interploidy—can affect rates of gene flow between lineages (see, e.g., Pinheiro et al., 2010). In the case of heterozygotic taxa, for major chromosomal rearrangements, such as fusions and fissions, a few plant studies (see below) and many more examples from animals point to a high level of hybrid sterility due to problems during meiosis (Lukhtanov et al., 2020). Thus, in *Ipheion*, species divergence was probably fostered also by a high degree of hybrid sterility reinforcing reproductive isolation mechanisms.

4.2 | Mechanisms of speciation and diversification within *Ipheion*

Speciation in plants often occurs by the gradual differentiation of populations in parapatry or allopatry, reinforced by the evolution of hybrid sterility (Yakimowski & Rieseberg, 2014). In sympatric

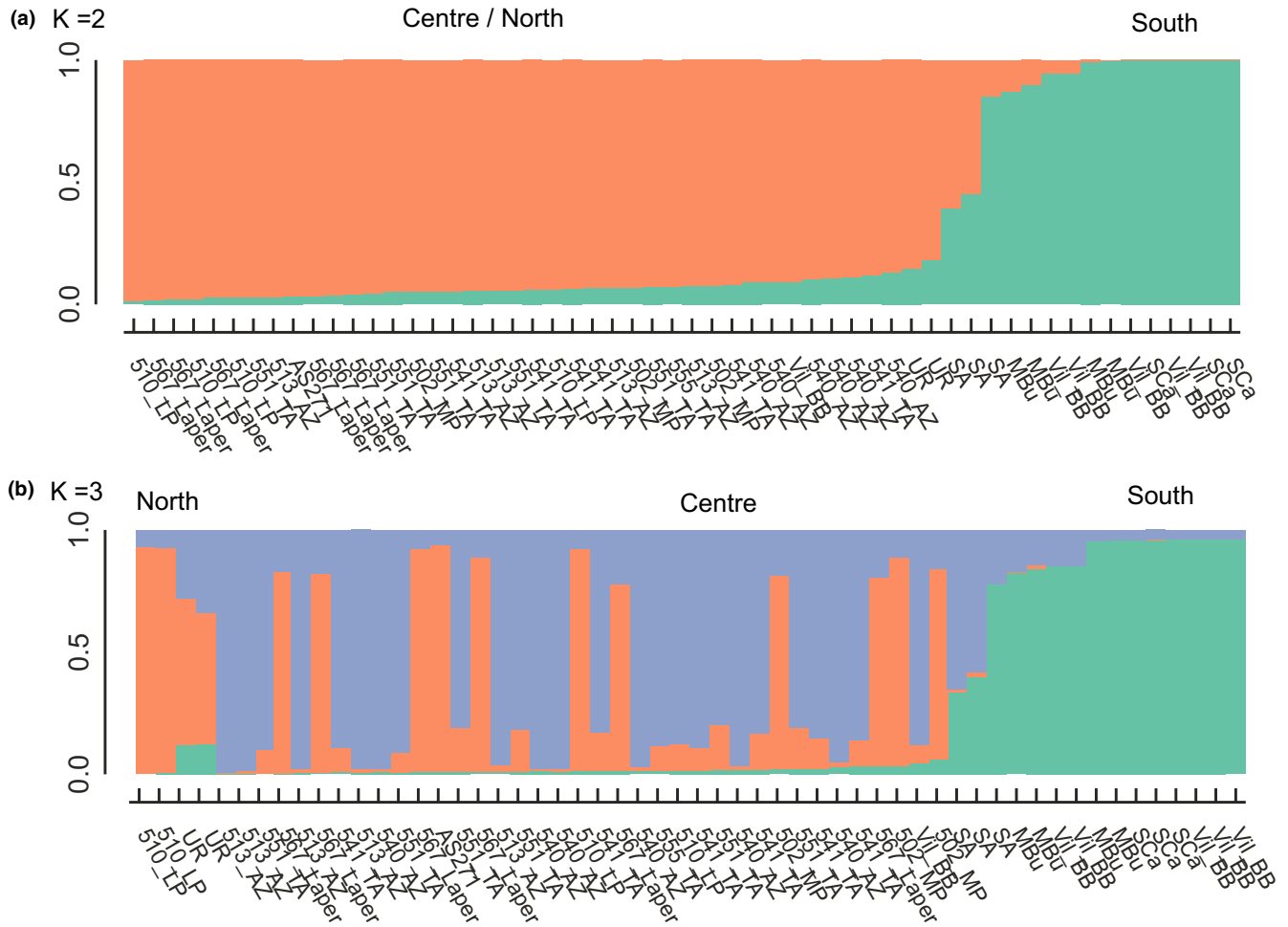

FIGURE 3 Population structure analysis of *Ipheion uniflorum* populations (56 specimens) with $K = 2$ (a) and $K = 3$ (b) based on 3,654 unlinked SNPs. Individuals are represented by a vertical bar that is divided by coloured segments representing the likelihood of membership to each cluster

TABLE 3 Pairwise F_{ST} values (lower bound – upper bound confidence interval limit) calculated according to Weir & Cockerham (1984) between the *Ipheion uniflorum* regions

	Centre (MP, LaPer, SA, AZ, TA)	South (BB, MBu, SCa)
North (UR-LP)	0.1268873 (0.1197123—0.1325643)	0.3179119(0.3094502–0.3253290)
Center (MP, LaPer, SA, AZ, TA)	—	0.2985968(0.2930035–0.3038498)

settings, speciation could only occur through the appearance of mutations resulting in incompatibility factors and barriers to gene flow among individuals (Rieseberg & Willis, 2007). The three *Ipheion* species currently co-occur in some areas of the Pampean region with no evident geographical barriers except perhaps for the widest rivers. The Uruguay River could represent such a geographical barrier to gene flow among plant populations, as in *Petunia axilaris* (Lam.) Britton, which has a similar distribution to *Ipheion* in the Pampas (Turchetto et al., 2014). However, in *Ipheion*, the Uruguay River only separates populations of *I. tweedieanum*. In *I. recurvifolium* it seems that the La Plata and Uruguay Rivers might have represented a barrier to dispersal because until now this species has not been recorded in Argentina. Nonetheless, the three species occur in sympatry and/or parapatry in Uruguay. However, we could not discard possible historical changes in the geology of the Rio de la Plata basin, and a possible role for its main rivers, as geographical barriers to gene flow and speciation within *Ipheion* (but see below). Barriers to gene flow might have resulted either as a consequence of gradual differentiation and cumulative effects of early-acting prezygotic reproductive barriers (such as ecogeographical morphotypes coevolving with different pollinators/mating systems) and the acquisition of divergent phenological and ecological traits leading to complete isolation, or by the appearance of late-acting postzygotic barriers to gene flow within species caused by gene changes or chromosomal rearrangements (Christie & Strauss, 2019; Rieseberg, 2001; Rieseberg & Willis, 2007).

Even though determining the order of emergence of reproductive incompatibilities and speed of evolution of reproductive barriers is difficult, diverse studies in plants show that barriers that occur early in the reproductive cycle contribute more to isolation than those taking place later (Christie & Strauss, 2019; Ramsey et al., 2003; Rieseberg, 2006; Sobel et al., 2019). In our phylogenomic analysis (Figure 2b), F_{ST} values indicate clear differentiation between species (>0.44), and the network analysis and genetic PCA of *Ipheion* species (Figure S2) points to the spontaneous establishment and long-term persistence of barriers to gene flow among populations of the three species despite their geographical proximity. Gradual differentiation and acquisition of early-acting (prezygotic) barriers among populations growing in sympatry or parapatry would have probably proceeded with recurrent gene flow, requiring thousands of generations before complete speciation (Christie & Strauss, 2018; Rieseberg & Willis, 2007; Sobel et al., 2019). Exceptions to this are hybrid and polyploid speciation, whereby species may acquire fully reproductive isolation in as few as one or two generations (Rieseberg, 2006; Ungerer et al.,1998).

Based on the current study and previous knowledge on the genus, the most parsimonious explanation for the current biological diversity and distribution of *Ipheion* species is that speciation occurred within *I. uniflorum* through fixation of karyotype novelties in natural populations, apparently caused by Robertsonian translocations (fission) and ploidy shifts. Such structural karyotype changes in both basic chromosome number and ploidy probably triggered the emergence of a rapid intrinsic postzygotic isolation via hybrid sterility that fostered later genetic, morphological, phenological and/or habitat diversification of the *Ipheion* lineages found in nature. This hypothesis was not only supported by the karyotype stability found within *Ipheion* species (Table S4) and the karyotypic and genetic differentiation observed among species in our study (Figure 2; Table 2), but it was also in agreement with previous karyological and cytological reports (Crosa, 1975; Souza et al., 2010).

In this context, the dynamics of the establishment of a small population of individual mates carrying the new karyotype remains unclear. Theory suggests that underdominance (i.e., when the heterozygous holds inferior fitness) will cause disruptive selection upon divergent karyotypes and, thus, it might magnify selective pressure upon minority individuals pushing them to the brink of extinction (see, e.g., Futuyma & Meyer, 1980). Chromosomal novelties might only persist when associated with strong selection against gene flow and/or through genetic bottlenecks, or in selfing populations, which are subject to genetic drift (Templeton, 1981). Yet, from an empirical viewpoint, underdominance may promote the local establishment of such karyotype novelties if it can influence mating direction and create bottleneck-like situations *in situ*, with novel and parental karyotypes coexisting without gene flow. Chromosomal rearrangements involving large chromosome segments are assumed to be rare in plants, but they are known to impede recombination and facilitate the establishment of rearrangements itself in nature (e.g., Kirkpatrick & Barton, 2006) as well as the accumulation of hybrid incompatibilities (Noor et al., 2001; Rieseberg, 2001). So, after appearance of the first individual carrying the rearranged chromosomes (such as those observed among *Ipheion* spp.), reduced recombination and underdominance may promote selfing or crossings within karyotypes and the formation of homozygous offspring. Alternatively or simultaneously, repeated formation of the same or similar chromosomal variants through hotspots of retrotransposable elements (Bourque et al., 2018) could also have facilitated the establishment of a small population carrying the novel karyotype. In the three *Ipheion* species, any other alternative hypothesis involving a gradual phenological and morphological differentiation of populations and the emergence of reproductive barriers before the karyological diversification would also imply that individuals carrying distinct (heterozygous) karyotypes (or evidence of introgressive hybridization among individuals having distinct karyotypes) should have been found within each species and/or within populations. Yet, this scenario is neither supported by our karyological results nor previous studies using individuals from distinct populations of *Ipheion* (Pellicer et al., 2017; Souza et al., 2010).

Karyotype changes are unlikely to spread through an established population simply because once introduced, such changes pose a selective disadvantage isolating the carrier from the parent population through hybrid sterility (Levin, 2002). The incidence of karyotype differences among all *Ipheion* species, in contrast to the sibling genus *Tristagma* in which the karyotype of all studied species has remained stable (Crosa, 1981; Sassone & Giussani, 2018), suggests that chromosomal rearrangements have played a key role in the diversification of *Ipheion* species, and perhaps in the origin of the genus, too. *I. uniflorum* and *I. tweedieanum* karyotypes are structurally similar and share the same fundamental number (the number of chromosome arms) of 14 with the genus *Tristagma* (Pellicer et al., 2017; Souza et al., 2010), suggesting that a Robertsonian translocation segregating ancestral populations was probably involved in the origin of the lineage and in shaping the later karyotype evolution in *Ipheion*. Molecular data using plastid and nuclear DNA markers indicated that *Ipheion* might have diverged from the Chilean–Patagonian genus *Tristagma* during the Late to Middle Miocene (Sassone & Giussani, 2018). This agrees with our results pointing to *I. uniflorum* as the species of the genus geographically closest to adjacent *Tristagma* taxa in northern Patagonia, and further supported by the southern *I. uniflorum* populations (BB, SCa, MBu) showing the highest genetic differentiation, which might indicate that they are oldest within the genus (Figure 3; Table 3).

Also, some individuals from the most differentiated populations of *I. uniflorum* shared genetic similarity to individuals from the population located in Uruguay (Figure 3b). The evidence overall, but particularly the genetic data, suggests that the southern populations of *I. uniflorum* (or its ancestor) diverged first and expanded to the north (Figure 2). During this expansion, the species have diversified going through at least two speciation events in Uruguay (Figure 2). The fact that during the Middle Miocene most of the current distribution of the genus was covered by the "Paranean Sea," except for the Pampas Mountains where the most differentiated *I. uniflorum* populations are found today (Ortiz-Jaureguizar & Cladera, 2006),

adds support to our interpretation. After marine transgression, the plains were dominated by grasslands, steppes and shrublands, which probably favoured the expansion of the remnant *Ipheion* populations into new geographical areas.

During the establishment of an incipient species, the evolution of hybrid sterility plays a key role (Christie & Strauss, 2019; Yakimowski & Rieseberg, 2014), and only strongly genetically isolated populations are likely to persist and speciate. In this scenario, chromosomal rearrangements are a mechanism enhancing homoploid speciation by partial or full reproductive isolation between the new and parental lineages. Supported by the genetic analyses, the karyotype of *I. tweedieanum* has probably originated after one Robertsonian translocation involving the fission of a submetacentric chromosome producing two acrocentric chromosomes (from x = 1 SM +5 A to x = 7 A), as previously suggested by Souza et al. (2010). Our data further suggest that speciation has been reinforced through changes in flower morphology and phenology accompanied by genetic differentiation and shifts in habitat adaptation. The origin of this rare species probably was in northwestern Uruguay (Figure 2b), and then it expanded to Entre Rios (Argentina).

In the case of tetraploid *I. recurvifolium*, previous authors (Crosa, 1975, 2004; Pellicer et al., 2017; Souza et al., 2010) proposed that it is a hybrid between *I. uniflorum* and a member of *Tristagma*. Even though *I. recurvifolium* exhibits floral similarities to *Tristagma sessile* (Phil.) Traub, this is more likely to be a result of convergent evolution of pollination strategies (Sassone et al., 2021). Our results support a unique history for *I. tweedieanum* and *I. recurvifolium* and we found no evidence of a hybrid origin or support for a shared genetic background with *Tristagma sessile* (Figure 2). The present data on geographical distribution, morphology, and karyotypic and genetic variability suggest that *I. recurvifolium* is an autotetrapolyploid (Figures 2, 3; Table 2), in agreement with previous cytogenetic data (Crosa, 1981; Souza et al., 2010). The *I. recurvifolium* karyotype differs from *I. uniflorum* by the absence of one acrocentric chromosome (Table S3). We hypothesize that *I. recurvifolium* probably arose from an ancestor of an extant *I. uniflorum* population from Uruguay that lost one chromosome arm accompanied by polyploidization. Genome duplication events have been often associated with chromosomal rearrangements (Levin, 2002). Moreover, our STRUCTURE analysis (Figure S5) shows *I. recurvifolium* has a cohesive gene pool with high gene flow among populations and no geographical differentiation (Figure S5), suggesting that the species probably arose after a single event via a structural chromosomal change followed by genetic and phenological divergence.

5 | CONCLUSIONS AND FUTURE PERSPECTIVES

Our study has provided genome-scale data of South American endemic garlics and offered a robust characterization of phylogenetic relationships within *Ipheion* and population structure, karyological and morphological evidence indicating the probable mechanisms responsible for their origin and diversification. *Ipheion* comprises three genetically isolated and morphologically well-differentiated species. The lack of barriers to gene flow in the Pampean region suggest that changes in the karyotype probably played a key role in the differentiation of *Ipheion* lineages. The first putative ancestral population of *Ipheion* was probably derived from one species of *Tristagma* that segregated through a chromosomal rearrangement in the south of the Pampean region. This entity became *I. uniflorum*, expanded northward and underwent at least two speciation events that resulted in establishment of *I. recurvifolium* and *I. tweedieanum*. We speculate that the establishment of these two lineages, reproductively independent from *I. uniflorum*, followed structural chromosomal changes and autotetraploidy in *I. recurvifolium*. The consequent formation of distinct karyotypes would have then imposed intrinsic postzygotic reproductive barriers, which were later reinforced by genetic, morphological (floral traits) and ecological (habitat, phenology) divergence.

Future analyses focused on calibrating in a more precise way the time of divergence of *Ipheion* from *Tristagma* as well as developing a time frame for the species of *Ipheion* by using biallelic markers and coalescence analysis will provide ages for points of species and population divergences and clues on the correctness of alternative speciation scenarios. Detailed studies identifying intraspecific karyotype variability, particularly among *I. uniflorum* populations, and divergence of genes in the rearranged and original chromosome segments will bring light to current evolutionary hypotheses and will help us to better understand how speciation through chromosomal structural changes proceeds in plants.

DATA AVAILABILITY STATEMENT

Sequence reads for the GBS Illumina runs were deposited in the European Nucleotide Archive (ENA, http://www.ebi.ac.uk/ena/data/view/PRJEB43919). Sampling locations, morphological matrix and georeferenced localities are provided as supporting information. All other data and scripts are available on dryad: https://doi.org/10.5061/dryad.v15dv41w5.

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AUTHOR CONTRIBUTIONS

A.S. and L.G. designed research and obtained funding. A.S. performed the research, and the genomic and morphological analyses. D.H. performed karyological analyses and J.B. helped with the analyses of GBS data. A.S. and D.H. wrote the paper with inputs from F.R.B. and L.G.

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