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RESEARCH

Phylogenetic, cytogenetic and morphological evidences are critical for recognizing a new genus: *Valdesiana,* an Iberian intergeneric allopolyploid between *Schenkia* and *Exaculum*

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Abstract

The present taxonomic status of *Schenkia elegans*, endemism recently described in the Iberian Peninsula, and its relationship with the sympatric and the nearest morphological species *Schenkia spicata* and *Exaculum pusillum* is revaluated. Different kinds of evidence based on plant morphology, ploidy estimation by flow cytometry, karyotype characterisation, and phylogenetic data have been analysed. Two maternally inherited plastid DNA regions (*trn*L intron and *trn*L-F spacer) and biparentally inherited nuclear ribosomal DNA sequence region (nrDNA ITS) have been used. Comparative multivariate analyses show an intermediate morphology of the *S. elegans* plants between the other two species studied. Flow cytometry and karyotype analyses in *S. elegans* point to an allopolyploid origin, with the latter constituted by a mixture of those of the diploids *S. spicata* and *E. pusillum*. Phylogenetic analyses based on plastid and nuclear DNA regions cluster *S. elegans* in two different clades, those of *S. spicata* and *E. pusillum*, suggesting a possible hybrid origin of *S. elegans* between both species, acting as maternal or paternal progenitors. In consequence, taking in consideration the taxonomic relationships among genera (*Exaculum*, *Schenkia* and the closely related genus *Zeltnera* found in America), a monotypic genus *Valdesiana* gen. nov. is proposed to accommodate the allopolyploid species, combined as *V. elegans*, for which immediate conservation measures must be evaluated.

Keywords Allopolyploidy · Endemism · Gentianaceae · New genus · Western Mediterranean

Introduction

Hybridization in plants is a highly important evolutionary process that favours adaptation to new or unstable habitats, as well as speciation, through the rupture or reinforcement of the reproductive barriers (Grant 1981; Rieseberg 1997; Whitney et al. 2010). Thus, interspecific hybridization could probably represent a source of adaptive genetic variation rather than mutation (Abbott et al. 2013). Hybridization can act in opposition to divergence, introducing an adaptive variation into a population, driving the development of stronger reproductive barriers, or generate new lineages and often creating reticulation patterns (Goulet et al. 2017).

Genome sequence analyses demonstrated that plant hybridization is an ancient evolutionary process (Alix et al. 2017), in which life history, pollination syndrome, breeding system, environment disturbance and genetic predisposition can be important drivers (Ellstrand et al. 1996). Whitney et al. (2010) showed that families and genera differed in hybridization propensity, being particularly common in rich and rapidly diversifying groups of organisms (Seehausen 2013), perhaps because of many young species in geographical proximity.

Hybridization occurs more frequently at the interspecific level. Nevertheless, many cases of intergeneric natural hybridization have been detected in several families of

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angiosperms (Webb and Druce 1984; Crawford et al. 1993; Weiblen and Brehm 1996; Saito et al. 2006; Soejima et al. 2008; Wu et al. 2010; Cetzal-Ix et al. 2012; Medan et al. 2012; Calvo et al. 2013; Desjardins et al. 2015; Smissen et al. 2015; Anghelescu et al. 2021; Onofre de Araujo et al. 2021). Whitney et al. (2010) found 3.5% of intergeneric hybrids in 3437 hybrids from 13 families, with a ratio higher in those groups with a high number of species.

Hybridization can occur between species sharing or not the same ploidy level, and in the latter, it is frequently accompanied by the subsequent full genome doubling, the hybridization between species or genetically differentiated populations of a species followed by genome duplication (allopolyploidy) could have important genetic and evolutionary consequences (Mable 2004; Mallet 2007; Soltis et al. 2009, 2016; Renny-Byfield & Wendel 2014; Sehrish et al. 2015; Fowler and Levin 2016). In any case, rapid chromosomal evolution and the availability of a suitable habitat for hybrids could favour hybrid speciation (Rieseberg 1997).

Genome duplications can produce a rapid process of reproductive isolation of the progenitors without the need for allopatry because they create a strong, although often incomplete, postzygotic reproductive barrier, and at the same time allowing the restoration of fertility more easily (Grant 1981; Otto and Whitton 2000; Coyne and Orr 2004; Rieseberg and Willis 2007). Likewise, polyploidy plays a predominant role in bursts of adaptive speciation and represents an important source of evolutionary novelty (Fowler and Levin 2016; Alix et al. 2017), by reproductive isolation, morphological differentiation, and deep effects on subsequent lineage evolution (Wood et al. 2009; Te Beest et al. 2012). These changes could be considered as revolutionary (Soltis et al. 2016), if both genetic and epigenetic changes are included. As a result, adaptation to new habitats and geographic areas is expected, with subsequent interactions with herbivorous animals and pollinators, thereby contributing to the emergence of new species (Stebbins 1950, 1985, 2014; Ramsey and Schemske 1998; Soltis et al. 2004; Thompson et al. 2004; Adams and Wendel 2005).

The frequency of genome duplication events throughout the history of seed plants and angiosperms attests to the important role that polyploidy has played in the evolution and diversification of plants in almost all vascular plant lineages (Soltis et al. 2009; Jiao et al. 2011; Wendel 2015; Barker et al. 2016; Alix et al. 2017; Sharbrough et al. 2017), especially in the case of allopolyploidy (Levin 1983; Coyne and Orr 2004; Mallet 2007). This could be explained because allopolyploids are more easily recognizable due to their phenotypic and molecular intermediacy or alternatively by the observation of transgressive phenotypes (Laport and Ng 2017).

The genesis and evolution of allopolyploids could be intricate. Many studies in species of Spermatophyta have evidenced that allopolyploidization events can be dynamic, repeated and that allopolyploid species may have multiple independent origins, as it has been proposed for several polyploids (Govindarajulu et al. 2011; Sigel et al. 2014; Neubig et al. 2015; Vallejo-Marín et al. 2015; Welles and Ellstrand 2016). At present, allopolyploidy represents an important main mechanism of diversification, with several new allopolyploids having originated just within the past century (Soltis et al. 2009).

The complex geological and climatic history of the Mediterranean region is key in favouring speciation processes (Thompson 2005), allowing the alternation of periods of isolation and contact between nearby species, thereby causing gene flow and genetic drift that increase the rate of diversification. In many cases, speciation has been accompanied by hybridization and polyploidy events (Vilatersana et al. 2000), which have been important sources for diversification in some Mediterranean lineages, especially in the Iberian, Italian and Balkan Mediterranean peninsulas (Sáinz Ollero and Moreno Saiz 2002; Vargas et al. 2009; Moreno Saiz 2011; Spaniel et al. 2011; Escudero et al. 2018). In the Iberian Peninsula, 13% of all taxa are considered as hybrid plants, and a 48.8% overall frequency of polyploidy have been reported in several genera (Marques et al. 2018).

In Mediterranean Gentianaceae polyploidy is a source of genetic diversity in genus *Centaurium*, with 15 polyploid taxa of the 27–30 recognized recently (Jiménez-Lobato et al. 2019), half of them or more suggested to have been originated by allopolyploidy (Ubsdell 1976; Mansion et al. 2005; Guggisberg et al. 2006).

Whitney et al. (2010) indicated for Gentianaceae a low propensity for hybridization, ranked in tenth place out of the 11 defined categories, although Löve (1953) previously indicated that the generic diversification in this family has been based in a high degree on allopolyploidy, as the high variation in the basic number of chromosomes between the different groups shows.

A molecular systematic study of the family Gentianaceae, based on the combination of the phylogenetic approach (*trnL* intron and *matK* sequence data), with support of morphological and cytogenetic data, concluded that one of the six monophyletic tribes recognized, Tribe Chironieae, is an important assemblage of 23 genera (Mansion and Struwe 2004). One of them, *Centaurium* Hill, resulted polyphyletic under its old or classic circumscription (Mansion and Struwe 2004), and consequently, genera *Gyrandra* Griseb. and *Schenkia* Griseb. were segregated, and a new genus, *Zeltnera* Mansion, was recognized (Mansion 2004).

The cytogenetic and phylogenetic analyses from nrDNA and cpDNA sequences (Mansion and Struwe 2004; Mansion and Zeltner 2004) indicate that *Zeltnera* (with 25 species restricted to the New World) constitutes a well-supported monophyletic assemblage, closely related to *Schenkia* (with

five Mediterranean Basin and Australian species) and the monospecific Mediterranean Basin *Exaculum* Caruel. The three genera share the morphology of the style with the two stigma lobes converging to form a subcapitate stigma and differ from the clear bifid style found in *Centaurium* (Mansion 2004).

Schenkia and *Zeltnera* differ in the type of the inflorescence, spiciform cyme in the first, but corymbiform or paniculate cyme in the second (Mansion 2004), whereas the anthers untwisted in *Exaculum* were the classic character used by many authors in Euromediterranean Floras for distinguishing it from those genera (Díaz Lifante and Valdés 2014).

Diploid level is present in *Exaculum* and *Schenkia*, although in the latter it is present only in one of the five species recognized, *S. spicata* (L.) G.Mans. This species shows a larger distribution area, from Mediterranean Region to West Asia (Mansion 2004), than *Exaculum*, which is found only in the West Mediterranean area.

However, every species of *Zeltnera* cytogenetically studied to date is polyploid, many of them showing a high incidence of dysploidy (Mansion and Zeltner 2004). More recently, a new species of *Schenkia*, *S. elegans* (Samp.) Z.Díaz, originally revealed as a race of *S. spicata*, was described (Díaz Lifante 2012), based on plants with both dichasial and monochasial cymoids, and flowers shortly pedicelled with subcapitate stigma. At present, this species has a very scattered area, limited to western Iberian Peninsula.

The current placement of *S. elegans* in the genus *Schenkia* is not entirely satisfactory because some morphological features resemble *Exaculum* (Díaz Lifante 2012), as the flowers pedicellate, not appressed against stem, calyx lobes unkeeled and erect, and usually 4-lobed corolla. Furthermore, the three Iberian species of these two genera, *S. spicata*, *S. elegans* and *E. pusillum* (Lam.) Caruel often cohabit in the same geographical area. In consequence, a hybrid origin for *S. elegans* could be hypothesised from these two species belonging to different genera. In this paper, morphological, cytogenetical and molecular analyses have been carried out in order to prove the possible hybrid origin for *S. elegans*, and its taxonomic consequences.

Materials and methods

Morphological analyses of plants

Detailed morphological multivariate analyses were conducted in wild plants of *S. elegans*, *S. spicata* and *E. pusillum* collected in 17 populations from Iberian Peninsula (Table 1, Fig. 1), most of them belonging to Southwestern region, where these taxa often coexist, and North Morocco. Measurements were obtained from five to ten pressed plants per population, except for four populations (Milfontes, Malalien, Abéjar and Águeda) of *E. pusillum* in which only one plant was measured. Vouchers are kept in herbaria (Table 1). Analyses at population level allowed an adequate choice of diagnostic characters, considering the variation within individuals and populations.

For examining relationships among the morphological characters and between individual plants, two multivariate ordination analyses were applied to a total of 34 characters (Table 2). A Principal Component Analysis (PCA) was used for 25 continuous characters. For analysing the adequacy of the set of 25 continuous characters to the PCA analyses, both the value for the Kaiser–Meyer–Olkin's parameter, and the significance level for the Bartlett's sphericity test were taken into account. An Optimal Scaling Analysis (OSA) was carried out for nine categorical characters. Analyses of variance (ANOVA) were made among the three taxa by comparing the mean values for each population of the continuous characters, for which previously normality and homoscedasticity were tested with Shapiro–Wilk's and Levene tests ($p < 0.05$). The multiple comparison post hoc tests of Bonferroni or T2-Tamhane (p less than 0.05), according to the homogeneity of variance, were used for comparing pairs of taxa. The statistic software *IBM SPSS Statistics 26.0* (IBM Corp. 1989, 2019) was used for all the analyses.

Furthermore, a morphological comparative analysis was made between plants of *S. elegans* and the type specimens of several species of *Schenkia* and *Zeltnera*, kept in the herbaria BM, BR, E, GH, HAL, JE, K, NY, P and PO [acronyms according to Thiers (2016)].

Cytogenetic analyses

Karyotype analyses

Chromosome counts were carried out in four populations of *S. elegans*, four populations of *S. spicata* and four populations of *E. pusillum* (see Table 1), for which genome size estimates were also obtained. Seeds were collected in several plants from natural populations and sown from January to March at room temperature in Petri dishes containing agar liquid as growth medium (0.125%). Previously, the seeds were surfaced sterilized by soaking them first for 1 min in a solution 10:1 distilled water/bleach (5.5% Sodium hypochlorite), and then, for 1 min in a solution of sterile distilled water. Root (sometimes stem) apical meristems of several seedlings from 5–10 parent plants were used for the somatic chromosome counts. Karyological method follows Díaz Lifante et al. (2009) except for the break of the mitotic metaphase, which was carried out by immersion of seedlings in distilled water at 0–1 °C for 12 h. The best well-spread metaphase plates were photographed with a Leika camera

Table 1 Populations and kind of taxonomic evidence investigated

Acronym[a]	Geographic origin and voucher[b]	Taxa studied[c]	Evidence analyzed[d]
ABE	1. SPAIN. Soria: Abejar, 41°52′49.24″ N, 2°43′46.83″ W, Aug 1966, *Segura Zubizarreta*, MA 360068	EXA	MOR
AGR	2. SPAIN. Granada, Agrón, 37°00′21.6″ N, 3°51′00.2″ W, Aug 2015, *Díaz Lifante, Andrés Camacho & Fuentes Carretero,* SEV 270233	SPI	FCM
ALG	3. SPAIN. Cádiz, Puerto Real, La Algaida, 36°32′12.0″ N, 06°12′33.0″ E, Jun 2015, *Viruel*, SEV 270232	SPI	FCM
ALM0	4. SPAIN. Huelva: El Almendro, 37°31′43.3″ N, 7°12′26″ W, Jul 2010, *Díaz Lifante and Girón*, SEV 288892 (EXA, ELE, SPI); ídem, Aug 2011, *Díaz Lifante & García Díaz*, SEV 270213 (EXA, ELE, SPI); SEV 249968 (ELE), SEV 249976 (SPI)	ELE, EXA, SPI	MOR, FCM, KAR, PHY
ALM1	4. SPAIN. Huelva: El Almendro bis, 37°31′43.3″ N, 7°12′26″ W, Jul 2016, *Díaz Lifante* and *García Llamas*, SEV 270234	EXA	FCM
ALM2	4. SPAIN. Huelva: El Almendro tris, 37°31′43.3″ N, 7°12′26″ W, Jul 2016, *Díaz Lifante* and *García Llamas*, SEV 270231 (EXA, ELE, SPI)	ELE, EXA, SPI	MOR, FCM
ATA	5. SPAIN. Sevilla: Coria del Río, Dehesa Atalaya, 37°14′31.76″ N, 5°59′43.55″ W, Jun 2016, *Díaz Lifante, Andrés Camacho and Díaz Fernández*, SEV 270230 (ELE), SEV 270230bis (EXA)	ELE, EXA	MOR, FCM, KAR
CAL	6. SPAIN. Tarragona: Segur de Calafell, 41°11′20.69″ N–1°36′33.17″ E, Aug 1976, *Rico*, MA 208490	SPI	MOR
CUA	7. SPAIN. Segovia: Cuatro Claros, 41°18′20.1″ N, 4°01′58.1″ W, Jul 2016, *Díaz Lifante & Andrés Camacho*, SEV 270235	SPI	FCM
DEH	8. SPAIN. Sevilla: Puebla del Río, Dehesa Abajo, 37°12′31.64″ N, 6°10'.45.5″ W, Jun 2016, *Díaz Lifante, Díaz Fernández* and *García Llamas*, SEV 270229 (ELE), SEV 270229bis (EXA); ídem, Aug 2016, *Díaz Lifante & Andrés Camacho*, SEV 288890 (SPI)	ELE, EXA, SPI	MOR, FCM, KAR
EMB	9. SPAIN. Huelva: Tharsis, Embalse Grande, 37°36′36.0″ N, 7°6′17.85″ W, Jun 2015, *Díaz Lifante*, SEV 288891	EXA	MOR, FCM, KAR
ESM	10. PORTUGAL. Beira Litoral: De Esmoriz a Mira, 40°48 N, 8°39 ' W, Jun 1901, *Sampaio*, PO-Samp	ELE	MOR
LIL	11. SPAIN. Toledo: Lillo, Laguna Altillo Grande, 39°41′39.4″ N, 3°18′3.6″ W, Jul 2016, *Mejías-Gimeno*, SEV 270236; July-2016, *Díaz Lifante and Andrés Camacho*, SEV 288886	SPI	MOR, FCM, KAR
MAL	12. MOROCCO. Malalien, 35°39′27″ N, 5°20′26″ W, Jun 1930, *Font Quer*, MA 92690	EXA	MOR
MIL	13. PORTUGAL. Baixo Alentejo: Milfontes, 37°43′29.30″ N, 8°46′54.69″ W, Aug 1905, *Sampaio*, MA 92698	EXA	MOR
MON	14. SPAIN. Soria: Embalse de Monteagudo, 41°23′7.1″ N, 2°11′2.4″ W, Jul 2015, *Díaz Lifante and García Escrivá,* SEV 288887	SPI	FCM
OFI	15. PORTUGAL. Beira Litoral: Playa de Ofir, 41°30′8.30″ N, 8°42′37.40″ W, July -2016, *Díaz Lifante and Andrés Camacho*, SEV 288888	SPI	FCM
PED	16. SPAIN. Valladolid: Pedraja del Portillo, 41°29′29.40″ N, 4°39′16.3″ W, Jul 2016, *Díaz Lifante and Andrés Camacho*, SEV 288889	SPI	MOR, FCM
PUE	17. SPAIN. Huelva: Puebla de Guzmán, 37°34′11.1″ N, 7°14′35.5″ W, Jul 1989, *Silvestre*, SEV 214292 (ELE); ídem, Jun 2015, *Díaz Lifante*, SEV 288894 (ELE and SPI), SEV 270203 (ELE), SEV 270207 (SPI), SEV 270208 (EXA)	ELE, EXA, SPI	MOR, PHY, FCM
SAG	18. SPAIN. Salamanca: Embalse del Águeda, 40°31′48.92″ N, 6°28′53.04″ W, Sep 1976, *Rico*, SALA 9505	EXA	MOR
SAL	19. SPAIN. Valladolid: Aldeamayor de San Martín, 41°30′41.57″ N, 4°38′19.01″ W, Jul 1983, *Ladero*, *Navarro* and *Valle*, SALA 4797	ELE, SPI	MOR
TIE	20. SPAIN. Huelva: Villanueva Cruces, La Tiesa, 37°36′37.6″ N, 7°5′35.3″ W, Jun 2002, *Díaz Lifante* & *Santa-Bárbara*, SEV 214423 (ELE); ídem, Jul 2010, *Díaz Lifante and Girón*, SEV 270211 (ELE), SEV 270211bis (SPI), SEV 288893 (ELE and SPI)	ELE, SPI	MOR
UPO	21. SPAIN. Sevilla: Campus de la Universidad Pablo de Olavide, 37°21′17.85″ N, 5°56′16.58″ W, Jun 2015, *Díaz Lifante*, SEV 270201 (ELE); Jul 2016, *Díaz Lifante* and *Andrés Camacho*, SEV 270201bis (SPI)	ELE, SPI	MOR, FCM, KAR, PHY

[a]Acronym used in the text and figures for the geographic origin. [b]Geographic origin and voucher (MA: Herbario del Real Jardín Botánico de Madrid; SEV: Herbario de la Universidad de Sevilla; PO-Sampaio: Herbarium of Sampaio in the Oporto University; SALA: Herbario de la Universidad de Salamanca) of the plants studied. Locations are numbered for their identification in map. [c]Taxon: *ELE*, *S. elegans*; *EXA*, *E. pusillum*; *SPI*, *S. spicata*. [d]Character nature: *MOR* plant morphology; *FCM* flow cytometry; *KAR* karyotype; *PHY* molecular phylogeny

applied to a Zeiss Axioscope microscope at a magnification ranged to 1800.

Conventional karyograms, with chromosome pairs arranged from largest to smallest, first by size and then by asymmetry, did not provide substantial diagnostic information. This is probably due to the very small size and the little differentiation among chromosomes, both in size and in asymmetry, which are gradually reduced. By this reason,

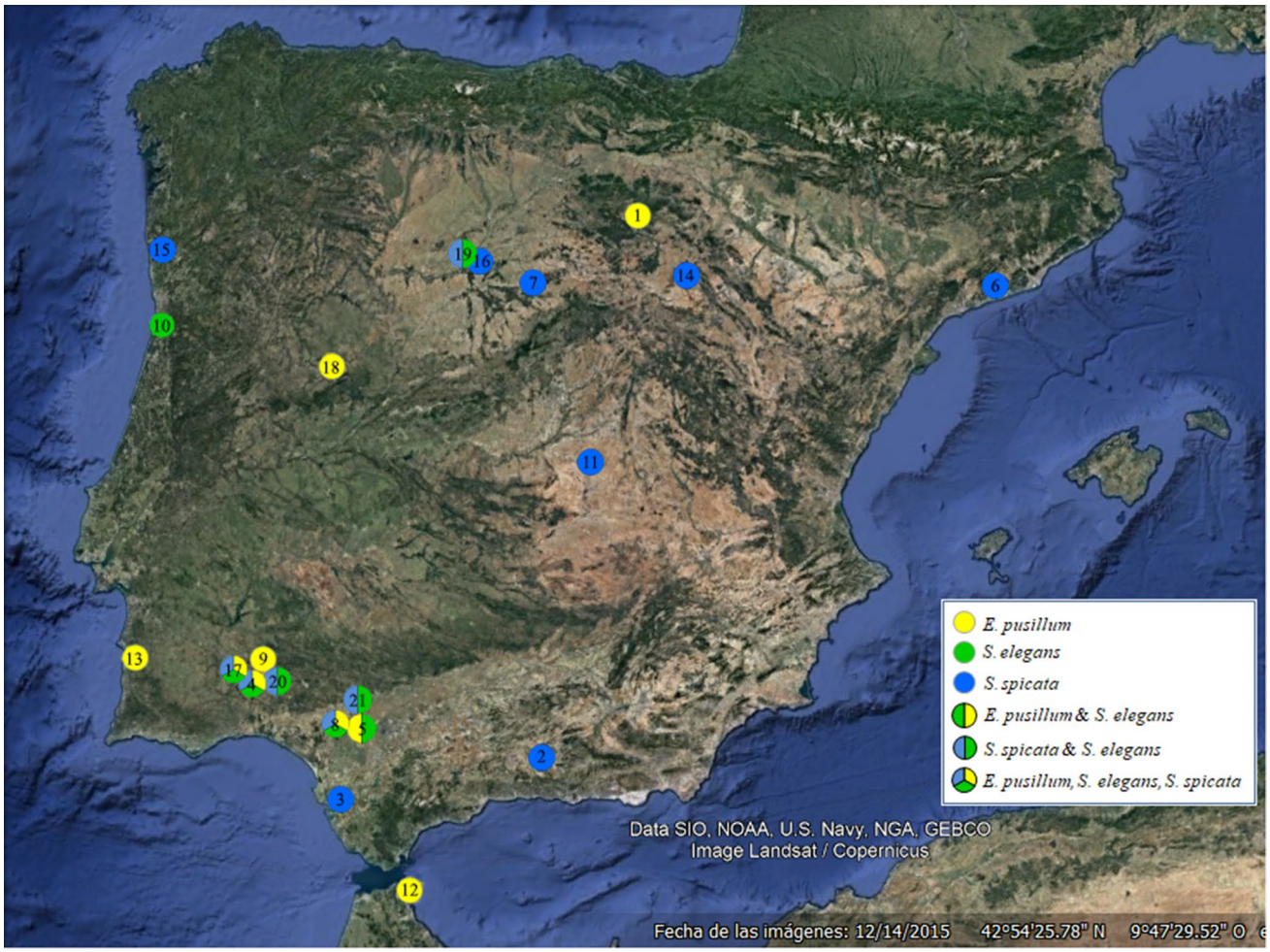


Fig. 1 Geographic localization of the populations analysed of *Exaculum pusillum*, *Schenkia elegans* and *S. spicata*. The colour of the dots denotes the species found in the population, in accordance with the legend. The locations have been numbered as indicated in Table 1. See abbreviations for the populations in Table 1. Source of map: Google Earth Pro 7.3.6.9345 (64 bit) 2020

the chromosomes for each taxon and population have been distributed in groups according to the length and asymmetry, from highest to shortest values, which facilitated the comparison of karyotypes.

Measurements of the chromosomes were made in the best plates obtained in the different populations studied: seven cells of *S. spicata* from two populations, eight cells of *S. elegans* from eight populations and nine cells of *E. pusillum* from four populations. The ImageJ 1.50i software (Wayne Rasband, National Institute of Health, USA) was used for it. The nomenclature of Stebbins (1938) and Levan et al. (1965) for the apparent size and morphology of chromosomes, respectively, was followed. The karyotype asymmetry was evaluated for each karyogram following Stebbins (1971), and by the indexes A_1 (intrachromosomal asymmetry, i.e. based on the ratio of the two arms) and A_2 (interchromosomal asymmetry, i.e. based on the chromosome size), as defined by Romero Zarco (1986).

Descriptive statistics (mean and standard deviation of the mean) were calculated for the genome size, the chromosome length (addition of the two chromosome arms), the ratio between the large to short arm of chromosome, and the A_1 and A_2 indexes of the karyotype asymmetry in each taxon. Univariate comparisons among the three taxa of the mean chromosome length, the total absolute length of the complement, the total length of complement referred to the haploid level and the A_1 and A_2 asymmetry coefficients were made.

Flow cytometry analysis

DNA content and ploidy level estimates were obtained using propidium iodide flow cytometric (FCM) analysis of nuclei isolated from silica-dried leaf tissues. A total of 119 samples, 33 for *E. pusillum* from seven populations, 35 for *S. elegans* from six populations, and 51 for *S. spicata* from 11 populations were analysed, including in average five plants

Table 2 Characters studied and abbreviations used in the text. States for categorical characters are specified

Categorical characters and their states	
Apex_lowLeaf	Apex of lower leaves:1, subacute; 2, subobtuse
Orient_Fl	Flower orientation:1, erect; 2, appressed against to the inflorescence axis
Sym_Calyx	Symmetry of the calyx:1, sepals equal and erect; 2 sepals unequal and twisted
Surf_Sep	Surface of the sepals:1, scabrid; 2, smooth
Keel_Sep	Presence of keel in the sepals:1, present; 2, present or absent; 3, absent
Col_Cor	Colour of the corolla:1, pale pink to pink, sometimes light yellow; 2, fuchsia
Num_lobCor	Number of the corolla lobes:1, 4; 2, 5
Apex_lobCor	Apex of the corolla lobe:1, obtuse and mucronulate; 2, acute or subdenticulate
Num_spirAnt	Number of helical turns of the anther:1, 0–1; 2, 2; 3, > 2
Continuous characters	
L_pl	Plant length (cm)
Degree_ramif	Degree of the stem branching (ratio of number of stem nodes branched)
L_lowLeaf	Length of the lower leaves (mm)
W_lowLeaf	Width of the lower leaves (mm)
L_upLeaf	Length of the upper leaves (mm)
W_upLeaf	Width of the upper leaves (mm)
L_cyme	Length of the principal cyma (cm)
L_intCym	Length of the first internode of the cyma (mm)
L_Brac	Length of the bract in the cyma's first flower (mm)
W_Brac	Width of the bract in the cyma's first flower (mm)
L_pedFl	Length of the pedicel in the cyma's first flower (cm)
L_Fl	Length of the cyma's first flower (excluding pedicel) (mm)
L_Sep	Length of the sepals (the longest, in case of inequality) (mm)
W_Sep	Width of the sepals (mm)
L_tubCor	Length of the corolla tube (mm)
L_lobCor	Length of the corolla lobes (mm)
W_lobCor	Width of the corolla lobes (mm)
L_Andr	Length of the androecia (from base of flower) (mm)
L_Stam	Length of the free part of the stamen filament (mm)
L_Anther	Length of the dehisced anthers (mm)
L_Pistil	Length of the pistil (mm)
L_Ov	Length of the ovary (mm)
W_Stig	Width of the stigma (mm)
L_Fr	Length of the capsule (mm)
L_Seed	Length of the seed (mm)

per population (Table 1). Fresh young leaves were collected, stored in hermetic plastic bags and kept in c. 4 °C during some hours. Once at the laboratory, the samples were dried with silica-gel. Measurements of the DNA content were made in samples of approximately one month old. Nuclei were isolated in a Petri dish containing 1 mL of WPB buffer (0.2 M Tris·HCl, 4 mM $MgCl_2 \cdot 6H_2O$, 1% Triton X-100, 2 mM EDTA $Na_2 \cdot 2H_2O$, 86 mM NaCl, 10 mM metabisulfite, 1% PVP-10; pH adjusted to 7.5 and stored at 4 °C; Loureiro et al. 2007) following the method described by Galbraith et al. (1983). For that, approximately 0.5 cm^2 of leaf tissue of our sample was chopped simultaneously with an equal amount of fresh leaf tissue of an internal standard using a razor sharp blade. The reference standard selected was *Bellis perennis* with a genome size of 2C = 3.57 pg calibrated with *Solanum lycopersicum* (2C = 1.96 pg, Doležel et al. 1992). Nuclear suspensions were filtered through a 50 µm nylon mesh and stained with 50 µg mL-1 of propidium iodide (PI, Fluka, Buchs, Switzerland). Then, 50 µg mL-1 of RNAse (Fluka, Buchs, Switzerland) was added to avoid staining of double stranded RNA. After 5-min incubation at room temperature, the relative fluorescence intensities of at least 1300 particles per G1 peak were analysed in a Partec CyFlow Space flow cytometer (532 nm green solid-state laser, operating at 30 mW; Partec GmbH, Münster, Germany). The results were obtained using FloMax software (Partec GmbH, Görlitz, Germany) as follows: fluorescence pulse integral in linear scale (FL) histogram; FL vs. time scatter-plot (to

assess fluorescence stability); FL vs. fluorescence pulse height scatter-plot (to remove duplets); and FL vs. SS in log scale scatter-plot (to evaluate effects of secondary metabolites; Loureiro et al. 2021). Polygon regions were defined in the FL vs. SS scatter-plot and further applied to the other plots to remove debris (Loureiro et al. 2021). Histograms were evaluated, retaining only samples with a coefficient of variation (CV) below 8% (silica-dried material often generated histograms of lower quality Čertner et al. 2021; Suda and Trávníček 2006); samples with higher CV values were discarded, and a new sample was prepared. The mean (± SD) CV values of the samples retained in this study were of 5.5 ± 1.1 (with minimum of 3.4 and maximum of 8.0).

A proxy of the holoploid genome size (2C) was calculated for each sample using the following equation: Sample 2C nuclear DNA content (pg) = (Sample G1 peak mean / Reference Standard G1 peak mean) * genome size of the Reference Standard. The nomenclature used for the DNA content and C-value followed Greilhuber et al. (2005), using holoploid genome size for the whole chromosome complement (2C) and monoploid genome size for the DNA content of the monoploid genome (1Cx). The monoploid genome size (1Cx) was calculated by dividing the holoploid genome size (2C) by the inferred ploidy level of each taxon in mass values (pg). Based on the genome size values obtained for the population characterized karyologically, diploids were identified to have genome sizes between 1.00 and 1.30 pg and tetraploids between 2.00 and 2.32 pg. The ploidy level was then assigned to all the populations analysed.

Molecular analysis

DNA extraction and molecular DNA regions

Dry leaf tissues from eight individual (Table 1) were used for genomic DNA extractions using Invisorb ® Spin Plant Mini Kit. Accessions from 27 species representing the main lineages of Tribe Chironieae (Gentianaceae) were downloaded from GenBank (Clark et al. 2016); see Supporting Information, Table S1). Specifically, accessions from the nuclear ITS1 and ITS2 regions and plastid *trn*L intron and *trn*L-F spacer were downloaded to be used in phylogenetic analyses. These accession sequences were first published by Struwe et al. (2002).

Sequencing

The nuclear ITS (internal transcribed spacer) DNA regions and the plastid DNA regions *trn*L intron and *trn*L-F spacer were amplified following protocols in Jiménez-Lobato et al. (2019). The PCR products were sequenced using Macrogene Europe Laboratory services.

Phylogenetic analysis

The sequences were aligned together with other sequences previously downloaded from GenBank using the software MUSCLE (Edgar 2004). Three ITS sequences of *S. elegans* showed a large number of additivities (ELE1-Alm, ELE2-Alm and ELE1-Puebla). The software jModelTest2 (Darriba et al. 2015) was used to infer the best possible model of nucleotide substitution (GTR + I + G was inferred as the best model in all analyses). The two matrices (cpDNA and ITS) were analysed using the phylogenetic software MrBayes (Ronquist and Huelsenbeck 2003; Ronquist et al. 2012). The analyses were run for 5 million of iterations, and the first 25% of the iterations were discarded as burn-in. The analyses were run twice using for MCMC chains to ensure we reach stationarity and convergence. The post-burn-in trees were used to build a consensus tree. The posterior probability (PP) was used as a measurement of clade support (Alfaro et al. 2003). Finally, an analysis combining the nuclear and plastid matrices was performed with the same parameters but for 10 million iterations.

Results

Plant morphology

Several morphological features in plant, leaves, flowers and fruits have been useful for distinguishing among *S. spicata, S. elegans* and *E. pusillum* after the multivariant analyses carried out, many of them shown in Fig. 2.

The indicators of the adequacy of the PCA are optimal for its application to the entire set of 25 continuous characters (values of 0.927 for the Kaiser–Meyer–Olkin's parameter and high significance for Bartlett's sphericity test, $\chi^2 = 3835.31$, $p < 0.0001$). Communality was equal to or higher than 0.7 in 22 characters. Five components reach eigenvalues greater than unity (S2), and the first three of them explain 69.40% of the variance. The first component achieves a high eigenvalue, retaining 55.03% of the variance, equivalent to almost 14 characters. In this component, 17 characters have eigenvector larger than 0.7, 12 of them with eigenvalues larger than 0.8. The length of the flower pedicel is the only character that correlates negatively with the rest. The other components retain little variance (< 8%). In the second component, the width of the stigma shows the higher eigenvalue, followed by the width of the lobes, and the length of the seeds and the first cyma internode. The width of the sepals shows eigenvectors values larger than 0.7 in the fourth component.

The two first principal components allow distinguishing the plants belonging to each taxon, placing them in three clearly discontinuous groups (Fig. 3a). No morphological

Fig. 2 Distinctive features among *Exaculum pusillum, Schenkia elegans* and *S. spicata* (from left to right): **a** branching pattern of the stem; **b** flowers, **c**; pedicel and calyx; **d** capsule; **e** half twisted anthers of *E. pusillum;* **f** fruiting flower and top vision of flower of *S. elegans;* **g** flower of *S. elegans* showing proximity between anthers and style. Scale bars: b and c, 2.5 mm; d, 2 mm; e, 0.4 mm

distinction is found for any taxon amongst the plants belonging to populations of different geographical regions. The first component represents many characters relative to the magnitude of different parts of the plant (plant height, leaf length and width, and length of sepals, corolla tube and lobes, stamens, anthers, ovary and pistil), many of them with high weight (up to 0.8) (Figs. 2a–c, e and 3b). This component allows differentiating *S. spicata* from *S. elegans* and *E. pusillum*, which shows the highest values and the greatest variance among the three taxa. Conversely, the lowest values for these characters are found in *E. pusillum*, whereas *S. elegans* shows an intermediate position, close to the origin of abscissa axis. The second component, based in fewer characters (seed size, width of corolla lobes and bracts), clearly separates *S. elegans* from *E. pusillum*, and rather well from *S. spicata* (Figs. 2b–c and 3b).

The OSA carried out with the nine categorical characters found values of 0.954 and 0.821 for the Cronbach's Alpha coefficients in the first and second dimensions, with, respectively, the 72.94% and 42.12% of the variance (S3). Three distant groups are differentiated in the ordination of the plants by the two dimensions originated, each one corresponding to a different taxon (Fig. 4a). Dimension 1 separates the populations of *S. spicata*, whereas dimension 2 distinguishes the populations of *S. elegans* from that of *E. pusillum*. The relationships of dependence and independence among the characters analysed show that the roughness and symmetry of the calyx, the arrangement of flowers and the shape of the corolla lobes were very discriminant in the first dimension (Figs. 2b–c and 4b). The apex of the lower leaves and the colour and number of the corolla lobes show medium values in the dimension 2 and somewhat lower

values in the first dimension. However, two characters, presence of keel in sepals and number of helical turns of the anther, reached similar and the highest values in the two dimensions (Fig. 2c, e–g).

A great inter-population homogeneity is found in *S. elegans*, whereas *E. pusillum* shows the highest dispersion of population data, with Atalaya (ATA) as the most variable population for the features analysed (4–5 dark or light pink corolla lobes) (Fig. 4a). The ordination of the states for the characters shows those exclusive for *E. pusillum*, as lower leaves acute, usually four corolla lobes (rarely five), which are white-yellowish or pale rose, and anthers non or with a helicoidal turn (Fig. 4b). Five states are exclusive for *S. spicata*: flowers appressed against to the cyma axis, unequal, twist and smooth sepals (Fig. 2c), corolla lobes acute or somewhat denticulate and anthers with 3–4 helicoidal turns. *Schenkia elegans* shares four states with *E. pusillum* (shaded in clear grey in Fig. 4b): obtuse lower leaves, erect flowers, corolla lobes subobtuse and relatively mucronulate, and equal, straight and more or less scabrid sepals (Fig. 2c, f–g). Three states are similar between *S. elegans* and *S. spicata*: usually 5 corolla lobes, which are subobtuse, and dark pink to fuchsia (shaded in dark grey in Fig. 4b). The only state exclusive for *S. elegans* was 1–2 helicoidal turns of the anthers, which really is intermediate between that of the other two taxa. Besides in *S. elegans*, the upper middle of the calyx lobes detaches from the corolla tube, whereas in *S. spicata* the calyx lobes applied totally, and in *E. pusillum* the character is variable, although this feature has not been included in the multivariate analysis.

The ANOVA for the morphological characters found significant differences among the population means at $p < 0.0001$ (2, 18 d.f.) for 23 of the 25 continuous characters of the three taxa (S4). However, no significant differences at $p < 0.05$ were found in the length of the first internode of the cyma and the width of sepals. Post hoc Bonferroni tests did not show significant differences at $p < 0.05$ between *S elegans* and *E. pusillum* in three characters (degree of branching, length of plant and length of cyma), and between *S. spicata* and *S elegans* in five characters (width of bract and corolla lobe, length of flower pedicel, and finally the fruit and seed).

However, the differences between the three taxa were significant at $p < 0.01$ in 10 characters (representing 40% of them), both for characters smaller than 2 mm and for characters greater than 2 mm (S5a and S5b, respectively).

Cytogenetic analyses

Chromosome number

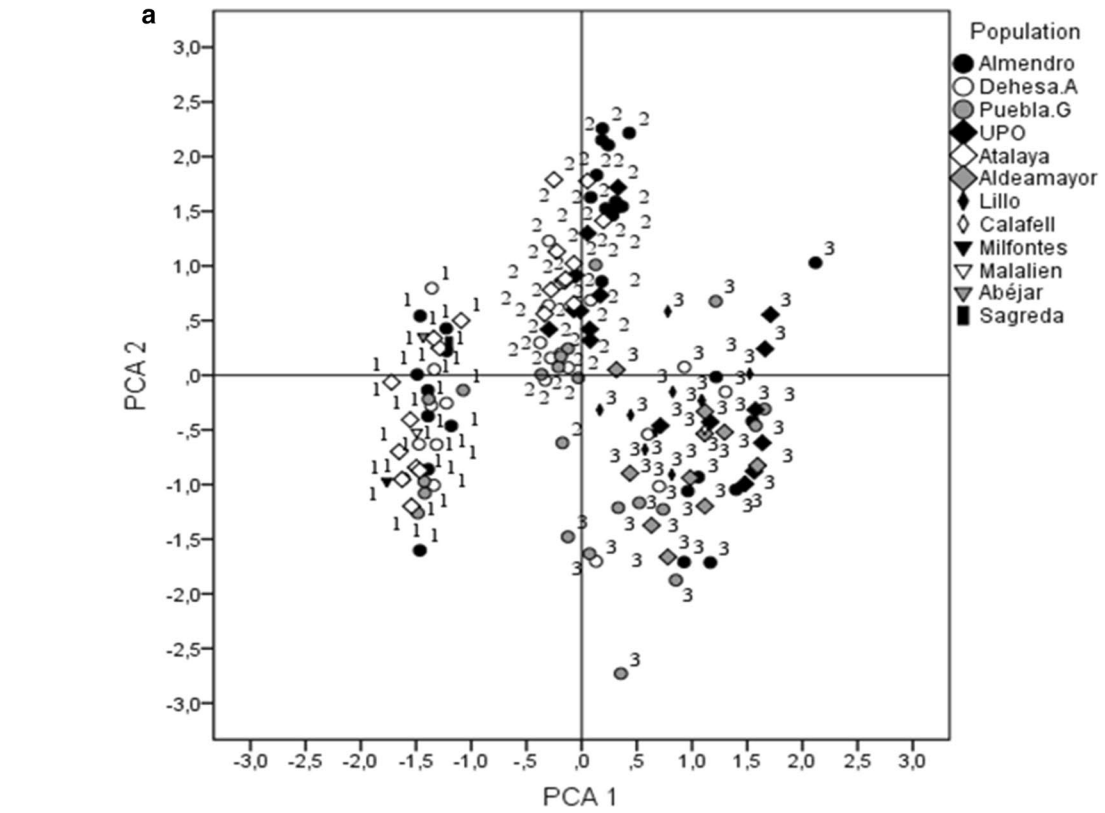
The somatic chromosome number $2n = 20$ is found in every seedling studied of *E. pusillum* belonging to 17 parent plants of four populations: three individuals from ALM0, five individuals from ATA, four individual from EMB, and five individuals from DEH) (Fig. 5a-b). In *S. spicata,* $2n = 22$ is counted in the seedlings of 13 parent plants from two populations (eight individuals from ALM0, and five individuals from LIL) (Fig. 5c-d). In *S. elegans,* a total of 33 parent plants from four populations (four of which from ALM0, twelve coming from ATA, six belonging to DEH and eleven from UPO) are studied, and $2n = 42$ is found in every seedling (Fig. 5e-g).

Chromosome size

The mean chromosome length reaches the highest value, 1.862 µm, in *E. pusillum*, followed by *S. spicata* with 1.709 µm, and *S. elegans* with 1.678 µm, but differences are not significant at $p < 0.05$ (Table 3). The total absolute length of the complement was 35.25 µm in *S. elegans*, almost twice as that of the other two taxa as can be expected from its polyploid condition (Table 3), although when this length is referred to the haploid level, differences are not found at 0.05 level ($F = 0.662$, $p = 0.526$; df = 2).

Karyotype morphology

According to the length and asymmetry of the chromosomes, three groups of them can be distinguished in the karyograms of the three species (Fig. 6). The first group is formed by large to medium sized metacentric (*M-m*) chromosomes (five pairs in *E. pusillum*, six pairs in *S. spicata* and 11 pairs in *S. elegans*). The second group includes three large to small sized submetacentric (*sm*) chromosome pairs, both in *E. pusillum* and *S. spicata*, and six of them in *S. elegans*. The third group is formed by two small sized pairs of metacentric (*M-m*) chromosomes, both in *E. pusillum* and *S. spicata*, yet four pairs in *S. elegans*. In several cells of *S. spicata* and *S. elegans*, one or two of the smallest chromosomes of the third group quite often show the two arms widely separated, probably due to the existence of a NOR region near to the centromeric region (see Fig. 5c–d, g). In consequence, the karyograms obtained in *S. elegans* seem to contain all the chromosome pairs present in those of *S. spicata* and *E. pusillum*.


a
Population
Almendro
Dehesa.A
Puebla.G
UPO
Atalaya
Aldeamayor
Lillo
Calafell
Milfontes
Malalien
Abéjar
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PCA 2
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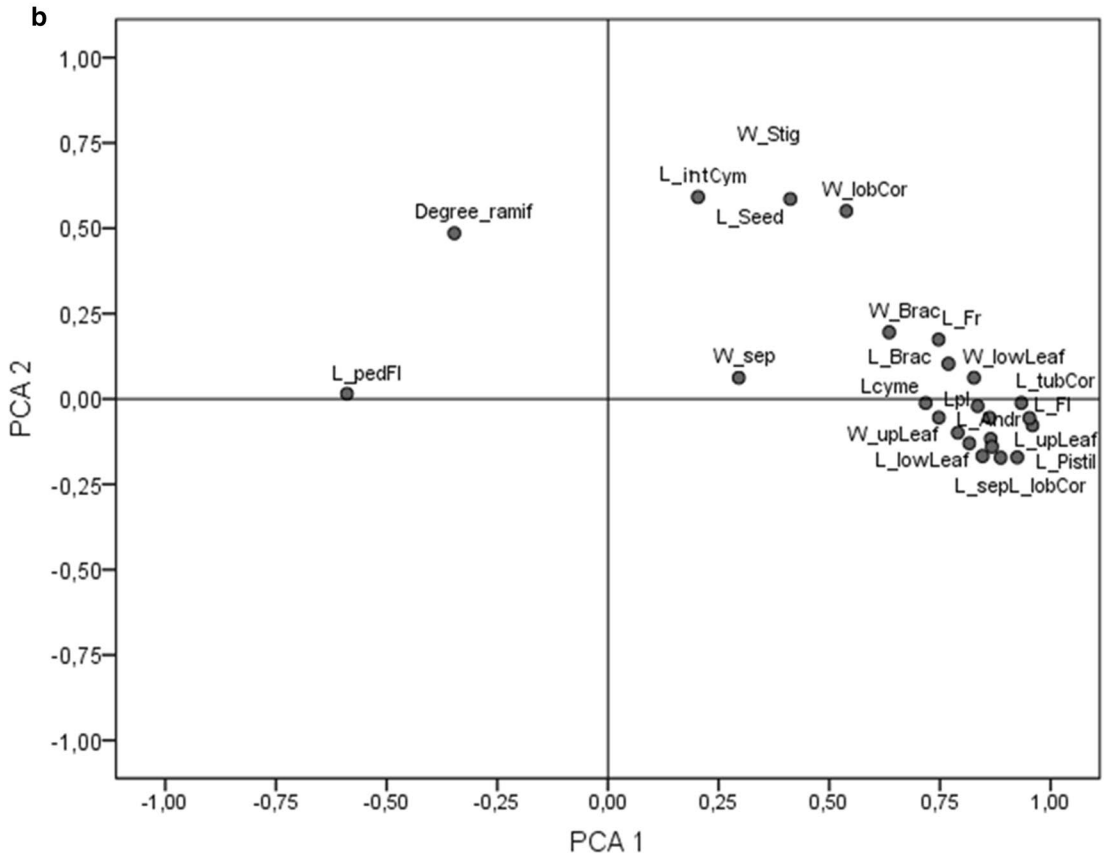
b
PCA 2
PCA 1
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0,75
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L_intCym
W_lobCor
L_Seed
Degree_ramif
W_Brac
L_Fr
W_sep
L_Brac
W_lowLeaf
L_pedFl
Lcyme
L_tubCor
L_Fl
W_upLeaf
L_upLeaf
L_lowLeaf
L_Pistil
L_sepL_lobCor

◂**Fig. 3** Results of the Principal Component Analysis based on the first two principal components (55.03% and 7.97% of variance): **a** ordination for the plants of the 12 populations of *Exaculum pusillum* (1), *Schenkia elegans* (2) and *S. spicata* (3); **b** ordination for the 25 continuous characters. See abbreviations for localities and characters in Tables 1 and 2, respectively

Karyotype asymmetry

Following classification of Stebbins (1971), the mean value for the length of the largest and the shortest chromosome pairs in the karyotype of the cells analysed are, respectively, 2.23 and 1.38 µm for *E. pusillum*, 2.19 and 1.35 µm for *S. spicata*, and 2.06 and 1.20 µm for *S. elegans* (S6). Thus, the range for the ratio between the length of the largest to the shortest chromosome is 0.478–0.665 in *E. pusillum*, 0.489–0.699 in *S. spicata* and 0.487–0.714 in *S. elegans*. These values meet with the subtype A2 of chromosomal asymmetry defined by Stebbins (1971), which point out 10–20% of the chromosomes with $r \geq 2$ in *E. pusillum*, 10% in *S. spicata* and 4.8–9.5% in *S. elegans*. Furthermore, regarding to the more precise asymmetry indexes A_1 and A_2 (Romero Zarco 1986), the ANOVA test finds only significant differences for A_2 ($F = 4.504$, $p = 0.024$; df = 2), although the post hoc Bonferroni test points out significant differences ($p = 0.038$) only between *E. pusillum* and *S. elegans*, with higher values in the latter (Table 3), whereas *S. spicata* does not distinguish from them.

Flow cytometry analysis. The mean values for the holoploid genome size found are 1.084 ± 0.064 pg in *E. pusillum*, 1.116 ± 0.039 pg in *S. spicata* and 2.098 ± 0.053 pg in *S. elegans* (Table 4). These plants are, thus, classified as having "very small" ($2C \leq 1.4$ pg) and "small" ($2C \leq 3.5$ pg) genome sizes following Leitch et al. (1998). Significant differences amongst taxa were obtained for the holoploid genome size ($F = 325.63$, $p < 0.0001$, df = 2), with *S. elegans* having significantly higher genome sizes than *E. pusillum* and *S. spicata* ($p < 0.05$). *Exaculum pusillum* and *S. spicata* have similar holoploid genome sizes ($p > 0.05$), and the diploid level is inferred for both taxa. *S. elegans* present holoploid genome sizes with almost the double of the other two taxa for every population studied (Table 3), and it is inferred as a polyploid, most likely a tetraploid. Assuming these ploidy levels, no significant differences ($F = 3.165$, $p = 0.063$, df = 2) are found among taxa in the monoploid genome sizes (0.542 ± 0.032 pg in *E. pusillum*, 0.556 ± 0.022 pg in *S. spicata* and 0.524 ± 0.013 pg in *S. elegans*, Table 3).

Phylogenetic molecular analyses

The new accessions for the plants here studied and obtained in this research are numbered as OQ848573 to OQ848580 for ITS, and OQ851735 to OQ851742 for *trn*L-F in GenBank. In the ITS tree (S7), the samples SPI-Alm (*S. spicata* from El Almendro) nests with the sample *S. spicata* from GenBank (PP = 1) and the sample EXA-Alm (*E. pusillum* from El Almendro) nests with *E. pusillum* from GenBank. The sample ELE1-UPO, ELE2-UPO and ELE3-UPO (all of them of *S. elegans* from the UPO population) groups with EXA-Alm (*E. pusillum* from El Almendro, PP = 0.93). The samples ELE1-Alm, ELE2-Alm and ELE1-Puebla (*S. elegans* from El Almendro and Puebla de Guzmán) are sister to the remaining samples of genus *Schenkia* (PP = 0.99) and grouped with samples from genus *Schenkia* (PP = 99). These three samples (ELE1-Alm, ELE2-Alm and ELE1-Puebla) are the ones with high number of additivities. These additivities displayed a pattern congruent with a mixture of ITS sequences from *S. spicata* and *E. pusillum*.

In the plastid tree (S8), the samples SPI-Alm (*S. spicata*) and ELE1-Alm, ELE2-Alm and ELE1-Puebla (*S. elegans*) are nested all together with *S. spicata* and *S. clementii* from GenBank (PP = 1). However, the samples ELE1-UPO, ELE2-UPO and ELE3-UPO (*S. elegans* from UPO) were nested with the sample EXA-Alm (*E. pusillum* from Almensilla) and *E. pusillum* from GenBank (PP = 1).

The ITS and plastid combined tree (Fig. 7) displays the simple SPI-Alm clustered in clade A with *S. spicata* and *S. clementii* from GenBank (PP = 1). The clade A is sister (PP = 1) to clade B (which includes the samples ELE1-Alm, ELE2-Alm and ELE1-Puebla). The clade C contains the samples EXA-Alm, and *E. pusillum* from GenBank, which is sister (PP = 1) to clade D (which contains the samples ELE1-UPO, ELE2-UPO and ELE3-UPO, PP = 0.95). The combined tree shows significant support for the relationships of genera *Schenkia*, *Exaculum* (including some samples from *S. elegans*) and *Zeltnera*. The three genera constitute a monophyletic clade (PP = 1). Genus *Zeltnera* is sister to the remaining genera (1) and genera *Schenkia* and *Exaculum* form a monophyletic clade (PP = 0.95).

Discussion

Morphological, cytogenetic, and molecular relationships amongst *S. elegans, S. spicata* and *E. pusillum*

Overall, multivariate morphological analyses confirm the recognition of three taxonomic entities clearly distinguishable by numerously continuous and categorical characters, and precise limits can be defined among them. Nevertheless, *S. elegans* is shown to be intermediate between *S. spicata* and *E. pusillum* in many of the morphological characters analysed, which might suggest a hybrid origin between these two species. The continuous traits show a gradient among

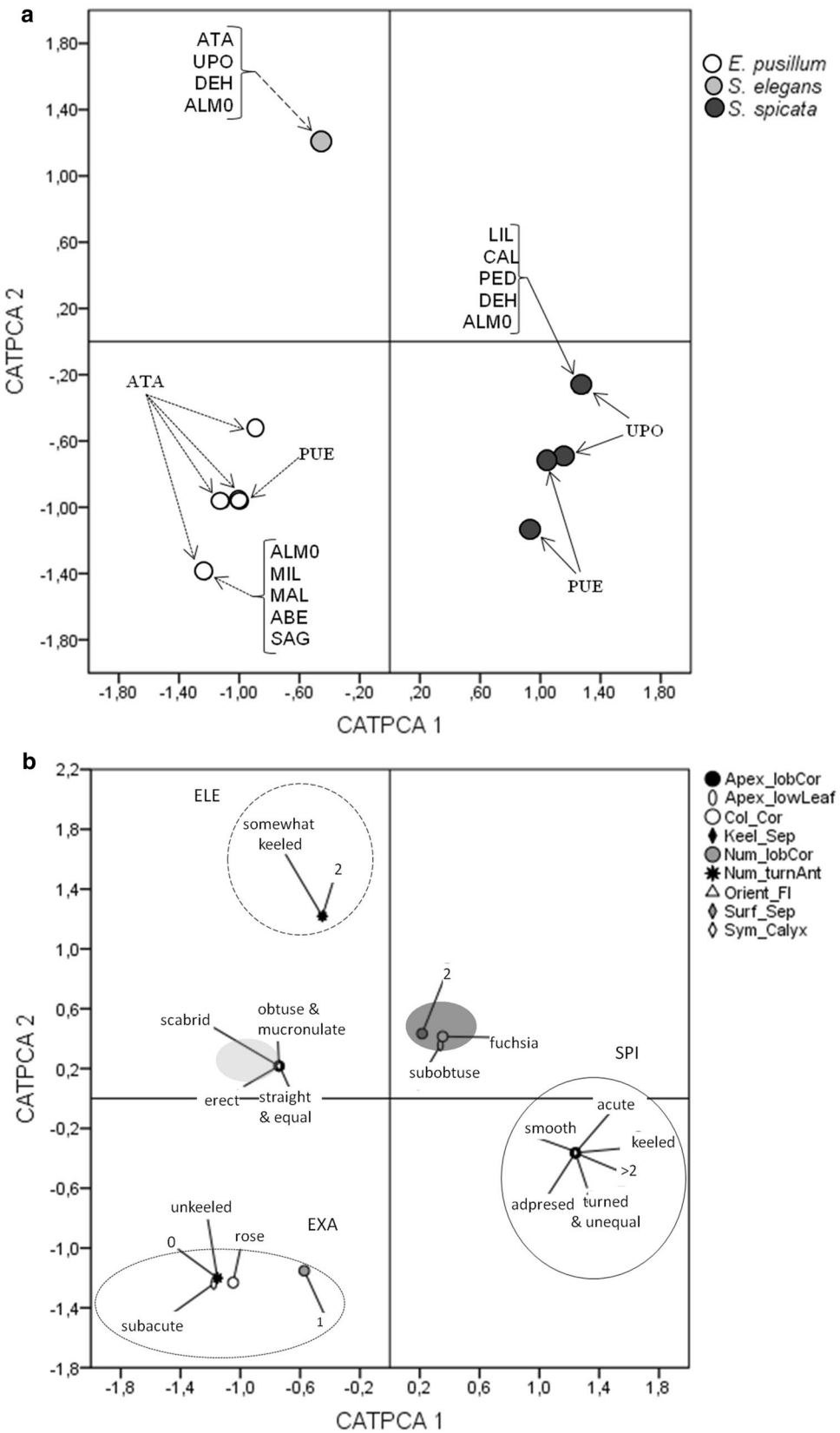

Fig. 4 Results of the Optimal Scaling Analysis (OSA) based on the first two dimensions (72.94% and 42.12% of variance): **a** ordination of the 12 populations; **b** ordination of the states of the nine categorical characters; exclusive states for the characters in each taxa are grouped (*ELE Schenkia elegans*, *EXA E. pusillum*, *SPI S. spicata*); shared states are grey-shaded. See abbreviations of localities and characters in Tables 1 and 2, respectively

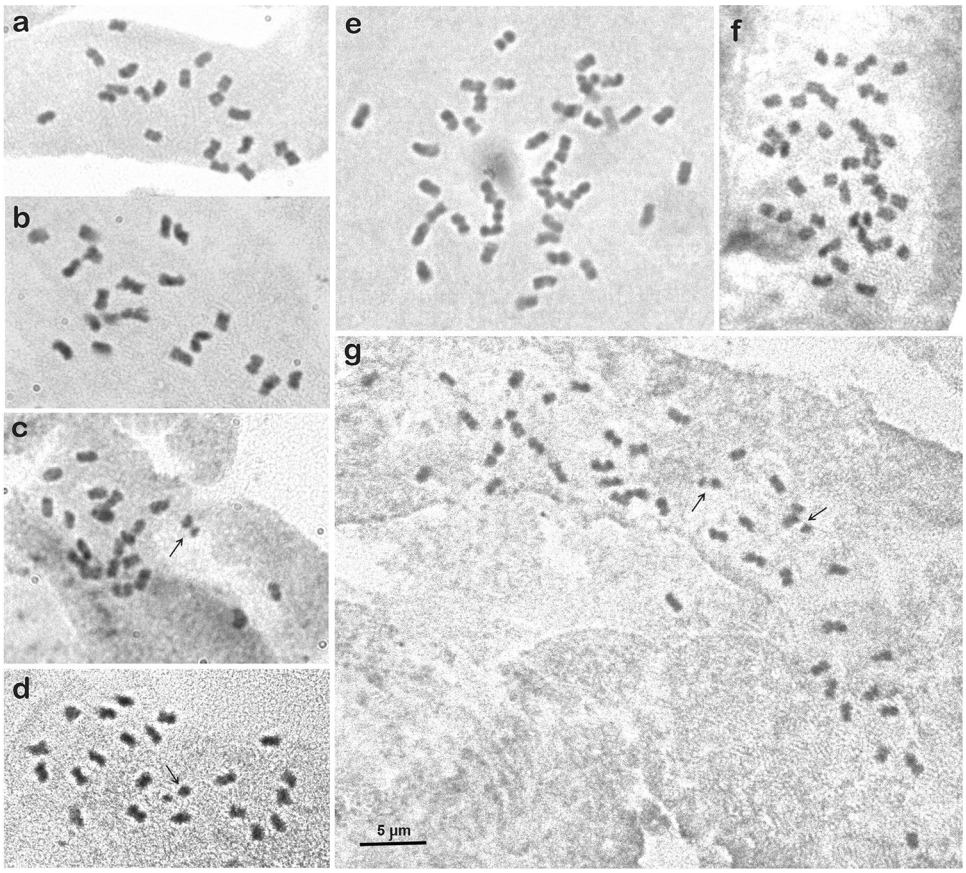

Fig. 5 Metaphase somatic plates: **a**–**b** *Exaculum pusillum*, $2n = 20$ (**a** Dehesa Abajo, **b** Dehesa Atalaya); **c**–**d** *Schenkia spicata*, $2n = 22$ (**c** El Almendro, **d** Lillo); **e**–**g** *S. elegans*, $2n = 42$ (**e** El Almendro, **f** Montequinto, **g** Dehesa Atalaya). Arrows show satellites. Scale bars: 5 µm (**a–g**)

Table 3 Characters summarizing the karyological constitution of the genome of *Exaculum pusillum*, *Schenkia spicata* and *S. elegans*. Range, mean, and standard deviation (s.d.) of values are included

Chromosome character	Taxon	Number of plants	Range (µm)	Mean (µm)	s.d.
Mean chromosome length	*E. pusillum*	9	1.477–2.206	1.862	0.2583
	S. spicata	7	1.542–1.875	1.709	0.1285
	S. elegans	8	1.340–1.959	1.678	0.2081
Total absolute length of complement	*E. pusillum*	9	14.77–22.06	18.61	2.5835
	S. spicata	7	16.96–20.62	18.79	1.4134
	S. elegans	8	28.14–41.14	35.25	4.3692
Total haploid length of complement	*E. pusillum*	9	7.383–11.03	9.308	1.2917
	S. spicata	7	8.479–10.32	9.398	0.7067
	S. elegans	8	7.034–10.28	8.812	1.0920
A1 index for karyotype intrachromosomal asymmetry	*E. pusillum*	9	0.206–0.366	0.3108	0.0497
	S. spicata	7	0.189–0.365	0.2969	0.0548
	S. elegans	8	0.184–0.361	0.2886	0.0580
A2 index for karyotype interchromosomal asymmetry	*E. pusillum*	9	0.081–0.198	0.1286	0.0346
	S. spicata	7	0.100–0.162	0.1301	0.0213
	S. elegans	8	0.136–0.196	0.1642	0.0203

the three taxa in the size of many structures of the stem, leaves and flowers (Fig. 2a–c), which could be expected for its hybrid nature. However, there are statistically significant clear discontinuities between the three taxa in their ranges of

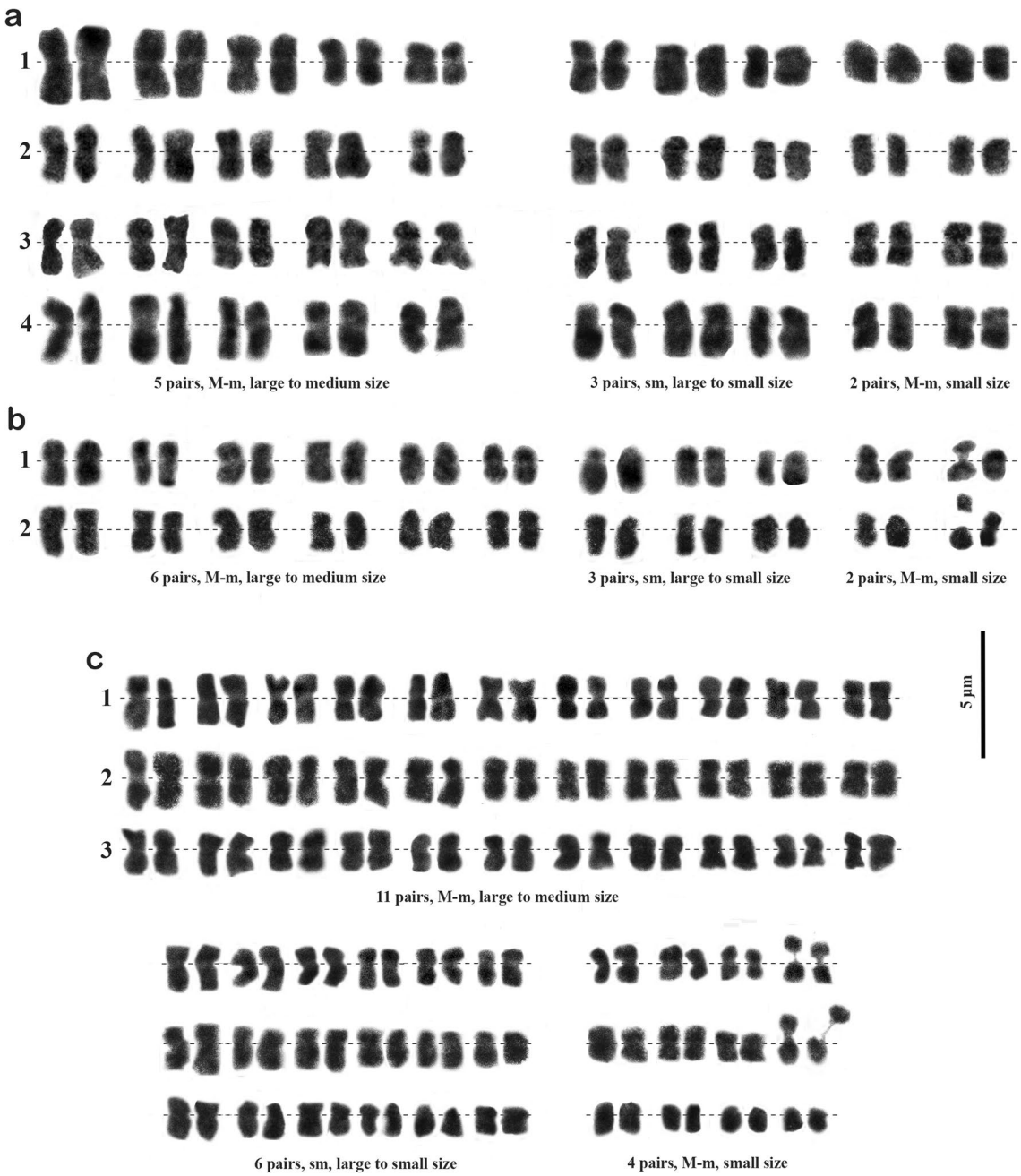

Fig. 6 Karyograms for several populations with chromosomes arranged by asymmetry and size in three groups in which they are arranged from largest to smallest: **a** *Exaculum pusillum*, $2n=20$ (1, Almendro; 2, Atalaya; 3, Dehesa Abajo; 4, Embalse Grande), **b** *Schenkia spicata*, $2n=22$, (1, Almendro; 2, Lillo), **c** *S. elegans*, $2n=42$ (1, Almendro; 2, Atalaya; 3, Montequinto). Scale bars: 5 µm (**a**–**c**)

variation for numerous continuous characters, with the highest interpopulation variability found in *S. spicata*. In relation to the categorical traits, *S. elegans* shows both parental character states, showing only one intermediate morphological discrete character, the 1–2 helicoidal turns of the anthers, which could be related to the length/width ratio of the anther. *S. elegans* did not show intermediate but higher values than those present in the other two taxa only in two continuous characters, size of stigma and seeds.

All the above indicates that *S. elegans* shows the pattern of differentiation that McDade (1990) and Rieseberg and Ellstrand (1993) propose for hybrids, that is, a mosaic of character states present in parents, identical to one or both parents, intermediate between the parental states, more extreme than the parental states, or novel. However, *S.*

Table 4 DNA amount estimations in *Exaculum pusillum*, *Schenkia spicata* and *S. elegans* by flow cytometry analyses in leaf material

Taxon	Population[a]	*n*	2C (pg)	1Cx (pg)	Ploidy level
E. pusillum	ALM0	3	1.023 ± 0.017	0.511	2*n* = 2*x*
	ALM1	9	1.198 ± 0.032	0.599	2*n* = 2*x*
	ALM2	5	1.081 ± 0.022	0.540	2*n* = 2*x*
	ATA	8	1.105 ± 0.020	0.553	2*n* = 2*x*
	DEH	5	1.070 ± 0.007	0.535	2*n* = 2*x*
	EMB	3	1.027 ± 0.022	0.513	2*n* = 2*x*
	Total		**1.084 ± 0.064**	**0.542 ± 0.032**	***2n = 2x***
S. spicata	AGR	4	1.066 ± 0.010	0.533	2*n* = 2*x*
	ALG	5	1.166 ± 0.055	0.583	2*n* = 2*x*
	ALM0	4	1.129 ± 0.075	0.565	2*n* = 2*x*
	ALM2	9	1.138 ± 0.044	0.569	2*n* = 2*x*
	CUA	5	1.137 ± 0.023	0.568	2*n* = 2*x*
	LIL	5	1.154 ± 0.086	0.578	2*n* = 2*x*
	MON	5	1.105 ± 0.078	0.528	2*n* = 2*x*
	OFI	1	1.124	0.564	2*n* = 2*x*
	PED	6	1.127 ± 0.040	0.563	2*n* = 2*x*
	PUE	2	1.110 ± 0.020	0.555	2*n* = 2*x*
	UPO	5	1.026 ± 0.020	0.513	2*n* = 2*x*
	Total		**1.116 ± 0.039**	**0.556 ± 0.022**	***2n = 2x***
S. elegans	ALM0	4	2.050 ± 0.036	0.513	2*n* = 4*x*
	ALM2	7	2.142 ± 0.094	0.536	2*n* = 4*x*
	ATA	10	2.095 ± 0.051	0.524	2*n* = 4*x*
	DEH	10	2.066 ± 0.042	0.516	2*n* = 4*x*
	PUE	1	2.180	0.545	2*n* = 4*x*
	UPO	3	2.054 ± 0.023	0.513	2*n* = 4*x*
	Total		**2.098 ± 0.053**	**0.524 ± 0.013**	***2n = 4x***

The sample size (*n*), mean values and standard deviation (s.d.) of the holoploid genome size (2C, in pg), the monoploid genome size (1Cx, in pg) and the inferred ploidy level are given for each population

Mean value and standard deviation for each species is given in bold

[a]Acronyms for populations in Table 1

elegans shows a low inter-population morphological diversity. As Seehausen (2013) indicated, the recombination of parent genomes after hybridization, causing break down of the genetic correlations, is expected when hybridization is followed by polyploidy. Thus, novel phenotypic characters can represent 'transgressive segregation' (Rieseberg et al. 1999) and contribute to the evolution of novel traits and hybrid speciation (Goulet et al 2017). The large stigmas and seeds could favour the self-pollination and seedling establishment, respectively, which furthermore would guarantee the stabilization and perpetuation of the genome of this annual species.

To our knowledge, this is the first estimation of the DNA content for *E. pusillum*, *S. spicata* and *S elegans*. Different DNA amounts between *S. elegans* and both *E. pusillum* and *S. spicata*, which is in accordance with the chromosome numbers found in these species. The chromosome number $2n = 42$ found in *S. elegans* differs from those of *S. spicata*, with $n = 11$ and $2n = 22$ (results herein and Mesquita Rodrigues 1953; Zeltner 1970, 1991; Carr 1978; Magulaev 1992; Mansion and Zeltner 2004), and *E. pusillum*, with $2n = 20$ (results herein and in Favarger 1960). Interestingly, in other non-Mediterranean species of *Schenkia* (*S. australis*, *S. clementii* and *S. sebaeoides*), $2n = 44$ has been reported (Carr 1978), which indicates a base number of $x = 11$ for the genus. On the contrary, wide karyological variability exists in the very closely related genus *Zeltnera*, in which $n = 17$, 20, 21 and 22 have been indicated, as a result of polyploidy and dysploidy (Broome 1976, 1977, 1978; Turner 1993; Mansion and Zeltner 2004). Moreover, the $2n = 42$ of *S. elegans* agrees with the chromosome number found in some species of *Zeltnera* belonging to the "Texan group" (*Z. maryanna* (Turner) Mansion and *Z. multicaulis* (Robinson) Mansion), and the "Mexican group" (*Z. marttinii* (Broome) Mansion, *Z. nudicaulis* (Engelmann) Mansion *Z. quitensis* and *Z. setacea* (Bentham) Mansion).

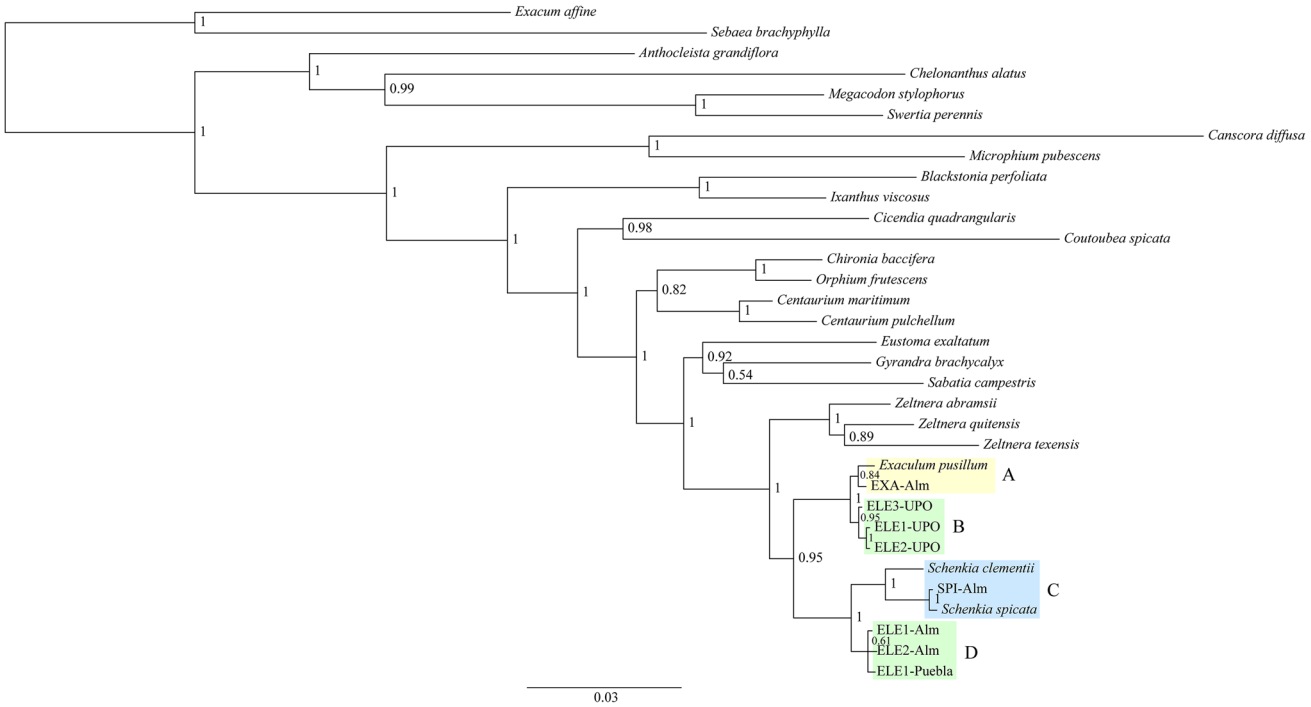

Fig. 7 Phylogenetic tree of tribe Chironieae, inferred using ITS and plastid sequences. The posterior probabilities (PP) are indicated to the right of the nodes. Clades A, B, C and D are differentiated. Colour squares represent the species: green from *Schenkia elegans* (ELE), blue for *S. spicata* (SPI) and *S. clementii*, and yellow for *Exaculum pusillum* (EXA). Samples represented with the whole specific name were obtained from the GenBank. For abbreviations of the populations sampled in this research, see Table 1

The karyograms reinforce the intermediate karyotype constitution in *S. elegans*, between that of *E. pusillum* and *S. spicata*. Despite *S. spicata* having two more chromosomes than *E. pusillum*, no significant differences were found in the DNA amount between *E. pusillum* and *S. spicata*, most likely because of the lower size of chromosomes in *S. spicata*. In *S. elegans,* the chromosomes are shorter than in *S. spicata* and *E. pusillum*, the karyotype shows a higher value for the A2 asymmetry index, and the DNA amount reaches lower values than it can be expected from the correlation between the mean genome size and the ploidy level. After the postpolyploid diploidization process, a "genome downsizing" mechanism, with extensive gene loss, is proposed (Soltis and Soltis 1999; Leitch and Bennett 2004; De Smet et al. 2013; Escudero and Wendel 2020). That mechanism itself could affect the expected correlation between the genome size mean and the ploidy level (Zenil-Ferguson et al. 2016).

Phylogenetic relationships among *Schenkia* (excluding *S. elegans*), *Exaculum* and *S. elegans* inferred from plastid DNA regions (*trn*L–intron—*trn*L-F spacer) revealed two divergent haplotype monophyletic groups for genera *Schenkia* and *Exaculum*. The same populations of *S. elegans* nesting in the same clade, that of *S. spicata* or that of *E. pusillum*, do so with both the plastid genome and the nuclear genome, which at the end reinforces their hybrid condition, following Funk (1985). Thus, assuming the maternal inheritance of plastid DNA as in most angiosperms (Smissen et al 2015), both *S. spicata* and *E. pusillum* could act as the maternal parent in the hybridization process. In addition, this points out to at least two potential independent evolutionary origins for *S. elegans*. Until now, *S. elegans* cohabits with both putative parental species in at least three populations, in which repeated hybridization events between the parent species occur. A similar pattern was found for example by Saito et al. (2006) in the F1 intergeneric hybrid genus *Crepidiastrixeris* Kitam.

Molecular evidence with nuclear and plastid DNA regions taken together indicates that *Exaculum*, *Schenkia* and *S. elegans* are very closely related and form a monophyletic group which is sister to the North American genus *Zeltnera* which in time probably has its origins in North America before the Pliocene (Mansion and Struwe 2004; Jiménez-Lobato et al. 2019).

Origin of *Schenkia elegans*

Every piece of evidence analysed in this study points out to an origin of *S. elegans* by allopolyploidy, with *E. pusillum* and *S. spicata* as the most probable parental species. Although these two diploid species occur in the Mediterranean Basin, the distribution area for *E. pusillum* (southern

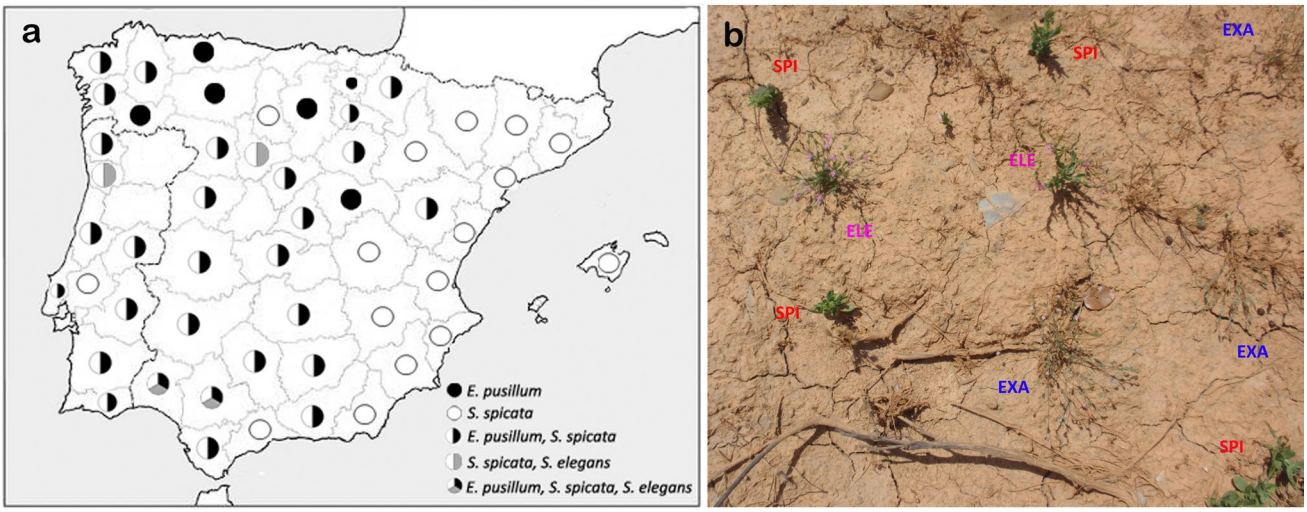

Fig. 8 **a** Distribution by provinces in the Iberian Peninsula of *Exaculum pusillum, Schenkia elegans* and *S. spicata*; **b** Close and intermixed arrangement of plants of *E. pusillum* (EXA), *S. spicata* (SPI), and *S. elegans* (ELE) in the locality of El Almendro (Huelva province)

and western Europe and northern Africa) is more reduced than that of *S. spicata*, which spreads up to western Asia, and was recently introduced in North America. The two species are occasionally sympatric in Western Iberian Peninsula (Table 1, Fig. 8a). In three of the populations here analysed, *S. elegans* grows together with *S. spicata* and *E. pusillum*, in other three only with *S. spicata*, and in one population only with *E. pusillum* (Table 1). Therefore, *S. elegans* has probably originated by repeated allopolyploidization at least twice, between the same putative parent species, a situation (polytopic hybrids) well documented and now considered the rule rather than the exception (Soltis and Soltis 1999; Mallet 2007; Leitch and Leitch 2008; Dixon et al. 2009; Soltis et al. 2014; Barker et al. 2016).

The putative diploid parents are morphologically and molecularly well differentiated, which could favour the reproductive stabilization of hybrids after allopolyploidy and the generation of a new species, as Grant (1981) and Stebbins (1985) proposed. Similar cases are found in Gentianaceae, as in some allopolyploid species of *Centaurium*, such as *C. bianoris* (Sennen) Sennen, *C. malzacianum* Maire, and *C. mairei* Zeltner (Mansion et al. 2005). On the other side, at present there is no evidence for introgression of *S. elegans* into its putative diploid parents. Furthermore, the great proximity between the anthers and the stigma (Fig. 2 f–g), the large size of this, and the very high fruiting rates (unpublished field observations) suggests that self-pollination may occur in *S. elegans*, although this must be demonstrated experimentally.

Due to the different diploid genomes of parents, a long-lived perennial growth habit or a self-breeding system is necessary to increase the chance of hybrid individuals producing polyploid offspring (Grant 1981), even more in the case of the allopolyploid species (Stebbins 1950). In consequence, reproductive barriers between polyploids and their diploid progenitors could originate (Rieseberg 1997; Husband and Sabara 2003; Fowler and Levin 2016; Glick et al. 2016; Siopa et al. 2020). Likewise, selfing can decrease the fitness disadvantages in the new polyploids caused by frequency dependent selection (*minority cytotype exclusion*; Levin 1975) and favours adaptation to new ecological niches or unstable habitats. It represents a reproductive guarantee in cases of low population density and scarcity of pollinators, and furthermore, it would allow safeguarding well-adapted genotypes, as well as their spatial differentiation. A combination of multiple factors related to pre-zygotic and post-zygotic reproductive barriers, and their potentially complex interactions, may also contribute to reproductive isolation (Widmer et al. 2009; Jersáková et al. 2010; Castro et al. 2020), which must be evaluated for *S. elegans*.

Although somatic doubling and triploid bridge are mechanisms producing polyploidy, the fusion of unreduced gametes from different genomes has been recognized as the most frequent mechanism enabler to allopolyploidy (Fowler and Levin 2016). The rate of production of unreduced gametes increases at higher levels of divergence between hybridizing parents, as does the fertility of polyploid hybrids (Mable 2004; Chapman and Burke 2007; Leitch and Leitch 2008).

The molecular analyses here enclosed show strong relationships among *S. spicata, S. clementii, S. elegans* and *E. pusillum*, with the morphologically nearest genus *Zeltnera*, when ITS and plastid DNA regions are combined. The divergence of *Zeltnera* from the clade *Exaculum-Schenkia* is dated by Mansion and Struwe (2004) in an earlier date (higher than 9.2 Mya) than that suggested by Jiménez-Lobato et al. (2019) (late Miocene, c. 7 Mya), during the

cooling and aridification of the global climate (Retallack 2004). Following Jiménez-Lobato et al. (2019), *Schenkia* and *Exaculum* separated shortly before the Messinian Salinity Crisis, about c.5 Mya. For Mansion and Zeltner (2004), the chromosome number of the species of *Zeltnera* agrees with a tetraploid constitution, probably originated from unknown diploid ancestors with $2n = 22$ and $2n = 20$, followed by chromosome rearrangements generating an intense dysploidy series ($n = 21$ to $n = 17$). Thus, *Zeltnera* seems to follow the evolutionary trend proposed by Levin (2020), in which initial polyploidy was followed by a wave of dysploid species formation. Although primary chromosome numbers meet with those present in *S. spicata* and *E. pusillum*, the origin of *Zeltnera* from hybridization between *Schenkia* and *Exaculum* does not seem possible, due to the very distant and disjunctive distribution areas of these taxa at present, and the relatively old origin of *Zeltnera* which predates the divergence of *Schenkia* and *Exaculum* (Mansion and Zeltner 2004; Jiménez-Lobato et al. 2019). On the contrary, the species *S. elegans* seems to have originated in more recent times, showing great morphological similarity between individuals from different populations. Thus, *Zeltnera* and *S. elegans* represent examples of the impact of polyploidy on plant diversification by its substantial contribution to the cladogenesis, as indicated by Wood et al. (2009), but in the case of *Zeltnera* a large later morphological and ecological diversification has been paired by with wide changes in the number of chromosomes.

The plastid sequences found in *S. elegans* are almost identical to those found in *S. spicata* or *E. pusillum*, and the ITS sequences found in *S. elegans* are almost identical to ITS from *E. pusillum* or similar to ITS from *S. spicata* including additive positions congruent with ITS mixing pattern with *E. pusillum*. These sequences with high number of additivities suggest that convergent ITS evolution is incomplete (which also suggests a very recent origin of this allopolyploid species), but more research is necessary to prove it.

Taxonomic revision

The names of the taxa must be given appropriately to stabilize nomenclature, so that it fulfils its main objective, which is to improve understanding of plant diversification processes and facilitate knowledge for conservation practices of a threatened or endangered singular taxon (Entwisle and Weston 2005; Knapp et al. 2004). Furthermore, the recognition of polyploids as taxonomic entities allows analysing their role in the evolution and in the ecological population dynamic (Laport and Ng 2017), having an impact on a better understanding of biodiversity and its conservation.

Taxonomic classification of polyploid complexes is difficult because their phenotypic differences ranging from subtle to markedly distinct, despite of generally strong reproductive incompatibilities. Unlike autopolyploids, allopolyploids are often well delimited, by their phenotypic and molecular intermediate characters, demonstrating an ecological, geographical and reproductive isolation, by which they deserve taxonomic recognition.

In the present taxonomic treatments, *Schenkia* and *Exaculum* constitute two genera well distinguishable morphologically and cytogenetically (Mansion 2004; Mansion & Struwe 2004) One of the five recognized species of *Schenkia*, *S. spicata* (= *Centaurium spicatum* (L.) Fritsch ex Janch.), is the most widely distributed and the only diploid. Much earlier Caruel (1886) described genus *Exaculum*. Since then, *Exaculum* has been widely recognized as a monospecific genus in all contemporary Euro-Mediterranean floristic works.

Mansion and Struwe (2004) and Mansion (2004) found that *Schenkia* and *Exaculum* diverged several million years ago (see also Jiménez-Lobato et al. 2019), and they differentiated *Schenkia* from the new genus *Zeltnera* by the inflorescence. Later, Díaz Lifante (2012) recognised *Centaurium spicatum* raça *elegans* Samp. at specific level as *Schenkia elegans* (Samp.) Z. Díaz, for plants with dichasial and monochasial cymoids, flowers shortly pedicelled and undivided style under the subcapitated stigma. The morphological and cytogenetic analyses showed in the present work point out *S. elegans* as a well-differentiated taxonomic entity, most likely originated by allopolyploidy from *S. spicata* and *E. pusillum*, as molecular data showed, which makes necessary to re-evaluate its taxonomic rank. Furthermore, *S. elegans* is very probably a fertile entity, as it has already commented above.

Morphological differentiation among S. elegans and the nearest phylogenetic genera

Exaculum differs from *Schenkia* by the paniculate inflorescence, constituted by dichasial or monochasial cymoids, with flowers largely pedicellate, very short and untwisted after anthesis anthers, rarely somewhat twisted, calyx lobes unkeeled and usually 4-lobed corolla. However, in *Schenkia* inflorescence is usually spiciform with flowers sessile or subsessile (although in some species as *S. clementii* is clearly pedicelled), twisted anthers, keeled calyx and 5-lobed corolla. In *Zeltnera*, genus sister to *Exaculum* and *Schenkia*, the anthers are always twisted, but a great morphological variability is found in the other characters: different shape and size of leaves, type of the inflorescence and cymes, length of pedicel, calyx lobes unkeeled or somewhat keeled, corolla 4–5-lobed, and size of the corolla, anthers and style (Mansion 2004). This variability makes difficult to establish generic limits between *Zeltnera* and these other two genera, although the molecular phylogenetic studies support a clear monophyletic group for *Zeltnera* (Mansion 2004).

Comparison between S. elegans and the other species of Schenkia

In *S. spicata,* the monochasial cymoids predominate, with a high number of distant flowers (5–20), sessile or with a very short pedicel, and appressed to the stem. In *S. elegans,* the stem is branched more often under the main cymoid, dichasial and monochasial cymoids are frequent, there are few flowers per cymoid (2–6) and flowers are distant, clearly pedicellate and divergent. Whereas in *S. spicata,* the calyx lobes are clearly unequal, bent and appressed to the corolla tube, in *S. elegans* they are subequal, straight and not fully appressed to the corolla tube. In *S. spicata,* the corolla lobes are ovate-lanceolate or narrowly elliptic, subobtuse with the apex entire or denticulate and nearly as long as the tube, whereas in *S. elegans* they are widely elliptic, obtuse, mucronulate and proportionally shorter than the tube. The style is more or less as long as the ovary in *S. spicata*, but it is clearly shorter in *S. elegans*.

In *S. australis* (Brown) Mansion, the flowers are occasionally somewhat pedicellate, the style is shorter than the ovary and the inflorescence is spiciform to racemose (holotype BM 000803703!, isotype K 000750973!). *Schenkia clementii* (Domin) Mansion has often monochasial cymoids as *S. spicata*, and longer pedicels (10–20 mm) and flowers (16–18 mm) than in *S. elegans*, in which, respectively, ranges of 1–2.2(4.5) mm and 7–11 mm are found. Some characters given for *Schenkia japonica* (Maximovicz) Mansion and *Schenkia sebaeoides* Grisebach, as wider basal leaves and fleshy leaves, respectively, clearly differentiate them from *S. elegans*. From a cytogenetic perspective, the genus *Schenkia* assembles diploid (*S. spicata* $2n=22$) and polyploid taxa (*S. australis*, *S. clementii*, *S. sebaeoides*) with $2n=44$ (Mansion 2004).

Comparison between S. elegans and Exaculum

Schenkia elegans resembles to *E. pusillum* in several characters, such as divaricate and often dichotomous branches, cymoids frequently dichasial with a low number of flowers per cymoid, flowers always pedicellate and detached from the stem, calyx lobes somewhat papillose-scabrid subequal and erect, not appressed to the corolla tube, and corolla sometimes 4-merous, with lobes widely elliptic and mucronate. However, in *E. pusillum* the very short anthers cannot twist after anthesis, and it has smaller flowers and anthers, and narrower leaves than *S. elegans*.

Comparison between S. elegans and Zeltnera

The high plant morphological variation enclosed in *Zeltnera* together with the cytogenetic and molecular evidence has allowed to recognize up to 25 species, distributed in three biogeographic groups (Mansion and Zeltner 2004) named as “Californian group”, “Texan group” and “Mexican group”. The analysis of the type specimens for the species of *Zeltnera* carried out in the present research showed that the plants of *S. elegans* more closely resemble some species of the “Mexican group”, as *Z. quitensis* (Kunth) Mansion (holotype:P-670841!; isotype:P-151799!) and *Z. stricta* (Schiede) Mansion (holotype:HAL-34829!), than those of the “Californian group” or the "Texan group". These two species share with *S. elegans* the absence of a leaf rosette and the presence of only one well developed stem, broad stem leaves, small-sized flowers (corolla and anthers), a shorter style than the ovary, stigma subcapitate and capsule oblongue to elliptic. However, *S. elegans* shows the corolla tube longer in relation to the lobes than these two taxa, and the pedicels significantly and persistently much shorter. Furthermore, the chromosome number is variable in both species, with both $n=21$ and 22 chromosomes found in each of them (Mansion and Zeltner 2004).

Overall, it is difficult to find diagnostic morphological features for *S. elegans* that clearly differentiate it from the highly variable genus *Zeltnera*. Morphologic, cytogenetic and phylogenetic evidence shows that *S. elegans* is a homogeneous taxon. However, *Zeltnera* encompasses a myriad of morphological diversity, with a wide combination of traits, as well as a great cytogenetic variability resulting from dysploidy, and a wide biogeographic and ecologic ranges its distribution area. Although *S. elegans* could be included within the wide morphological diversity of *Zeltnera*, both taxa have completely different phylogenetic origins, widely separated in time and in different biogeographic contexts.

There are three possible taxonomic solutions here that could reconcile the evolution of the four recognized main lineages: (i) follow a morphological approach and merge *Zeltnera*, *Exaculum*, *Schenkia* and *S. elegans* in a single genus *Schenkia*, (ii) merge *Exaculum*, *Schenkia* and *S. elegans* in a single genus *Schenkia*, or (iii) follow the phylogenetic evidence and describe a new monotypic genus for *S. elegans*. Following the recommendations of Stuessy (2009) for remodelling genera, to consider an unique genus *Schenkia* for *Zeltnera, Schenkia, Exaculum* and *S. elegans* would be inadvisable from a practical point of view and a theoretical perspective, and it would obligate to make numerous new combinations for the 25 species of *Zeltnera*, which would increase the list of indexed names in databases and create confusion among the users of classification with conservation purposes. Only one nomenclatural change would occur if *Exaculum, Schenkia* and *S. elegans* are including in genus *Schenkia*. However, in *Exaculum* a high number of autapomorphies in the flower characters have accumulated, which has increased its morphological differentiation and a high ecological specialization, reinforcing its biological unit. Besides, from a practical point of view for users of

classification, there is an old and long tradition of recognizing *Exaculum* as a highly different and monotypic genus in Mediterranean flora (Díaz Lifante and Valdés 2014). The phylogenetic data (Mansion & Struwe 2004) indicate that this is a very old and distinctive lineage that diverged long time ago from *Schenkia* and *Zeltnera.* Moreover, the fact that *S. spicata* and *E. pusillum* can hybridise only proves the existence of a common ancestry between both genera, although it does not force to place them in a unique genus. As McKenzie et al. (2004) stated, "A rigid requirement for cross-incompatibility between genera would result in fewer, larger genera that might obscure rather than clarify phylogenetic relationships".

The three taxa share a wet habitat, but there are certain differences both in the phenology and in the substrate they occupy. *Exaculum pusillum* is found in community of therophytes in the margin of streams and seasonal ponds, in humid and acid substrates (siliceous sands, slates) which is recognised as *Isoeto-Nanojuncetea* association. *Schenkia spicata* has a wider distribution area, also in margin of streams and seasonal ponds, when they have dried. It preferably inhabits basic soils and somewhat salty, as in littoral ponds, but it can be found also on acid substrates. *Schenkia elegans* occupies the margin of depressions, coastal or inland, as well as the dried channels of ponds and temporary watercourses, on acid (Andévalo, Bajo Guadalquivir), or basic (Beira litoral) substrates. Nevertheless, there is a phenological difference, *S. spicata* flowers later than *S. elegans* and *E. pusillum*. Accordingly, the monotypic genus is the best solution for us and we propose to consider a new genus to accommodate the allopolyploid entity *S. elegans*. This decision is supported by the existence in *S. elegans* of an intermediate, but not overlapping, range for many quantitative continuous features which differentiates it clearly from *Exaculum* and other *Schenkia* species, two of them exclusive (width of stigma and length of seeds), and one discrete (1–2 helicoidal turns of the anthers).

As a result of this research, and in order to achieve stability for the classification, here a new genus, *Valdesiana*, is described for the well-established Iberian endemism *S. elegans*, in the important diversification centre of Mediterranean Region, which could represent the origin of an emerging evolutionary lineage.

Taxonomic treatment

Key to the most phylogenetically related genera

1a Style slightly bifid; capsule subcylindric, oblongoid, or ovoid-oblongoid ..... ***Centaurium***

1b Style with the two stigma lobes converging to form a subcapitate stigma; capsule ellipsoid to ovoid **2**

2a Anthers not helicoidally coiled, or at most twisted only half a turn ..... ***Exaculum***

2b Anthers helicoidally coiled after anthesis **3**

3a Inflorescence spiciform, sometimes racemose, often formed by monochasial cymes. Flowers usually sessile to subsessile, rarely pedicellate. Calyx lobes keeled; corolla usually 5-lobed ..... ***Schenkia***

3b Inflorescence corymbiform, paniculate, racemose or capituliform, formed by both monochasial and dichasial cymes. Flowers subsessile or pedicellate. Calyx lobes unkeeled or slightly keeled; corolla 4–5-lobed **4**

4a Flower pedicels (2) 3-25 (50) mm ..... ***Zeltnera***

4b Flower pedicels 1-2.2 (4.5) mm ..... ***Valdesiana***

Valdesiana Z.Díaz & M.Escudero, **gen. nov.**—TYPE: *Valdesiana elegans* (Samp.) Z.Díaz & M.Escudero, **comb. nov.**

Eponymy: The name of the genus is dedicated to Prof. Benito Valdés, eminent and indefatigable Mediterranean botanist who developed important projects of Flora and Taxonomy, and trained a good number of taxonomists at the University of Seville.

Diagnosis: Herb, annual, uniacaule. Basal rosette of leaves absent. Cauline leaves ovate-elliptical or elliptical, acuminate. Flowers (4)-5 merous, not appressed to the stem, arranged in lax and compound dichasial and monochasial cymes. Calyx lobes (4)-5 equal, rarely subequal, somewhat keeled, mucronate. Corolla with (4)-5 lobes, 1/3–1/2 tube length, obtuse, mucronulate, rose-purple, rarely white. Anthers helicoidally coiled in 1–2 turns after dehiscence. Ovary superior, sessile, ovoid-ellipsoid; style marked, 1/2–2/3 of the ovary length, bifurcate very shortly in the upper part; stigma lobes entire, round, convergent in anthesis, subcapitate.

Description: Herbs, annual, uniacaule, with sympodial branching, glabrous. Stem erect, branched in the upper part, sometimes also in the lower part. Basal rossette of leaves absent; middle and upper leaves ovate-elliptical or elliptical, acuminate, with 3–5 nerves. Inflorescence cymose, formed by compound cymes, with dichasial branching in the proximal part and monochasial in the distal part, sometimes with some intercalar dichasial branch, very lax. Flowers pentamerous, rarely tetramerous, almost sessile or shortly pedicelled, separate from the stem. Calyx lobes (4)-5 equal, rarely subequal, not exceeding or exceedingly somewhat the corolla tube, rather keeled, smooth or somewhat scabrid, mucronate, separate and not applied to the corolla tube. Corolla hypocrateriform; lobes 4–5, 1/3–1/2 tube length, entire, obtuse, mucronulate, patent, rose-purple, rarely white.

Fig. 9 Lectotype of *Centaurium spicatum* raça *elegans* Samp. (PO-6749 G.S.): First specimen on the right

Androecium with 4–5 stamens, erect, moderately converging in anthesis; exceeding or equalling the stigma; anthers slightly sagittate in the base, helicoidally coiled in 1–2 turns after dehiscence. Ovary superior, sessile, ovoid-ellipsoid, and style defined, 1/2–2/3 of the ovary length, slightly bifurcate in the upper part, each branch widened in a stigma lobe; stigma lobes entire, round, convergent in anthesis, subcapitate, with long papilles, yellow, reaching or exceeding somewhat the mouth of the corolla tube. Fruit an ovoid-ellipsoid capsule.

Valdesiana elegans (Samp.) Z.Díaz & M.Escudero, **comb. nov.** ≡ *Centaurium spicatum* raça *elegans* Samp., *Man. Fl. Portug.*: 383 (1913), basion. ≡ *Schenkia elegans* (Samp.)

Z.Díaz, *Flora Iberica* 11: 85 (2012).—LECTOTYPE: (**designated here**): Ílhavo: Ria, 30 Jun 1901, *G. Sampaio* (PO-6749 G.S. [photo]!). (Fig. 9). Ind. loc.: "Prados maritimos, de Esmoriz a Mira".

Etymology: The name refers to the slender stems and thinner branches than the type of *Centaurium spicatum*.

Lectotypification: Sampaio (1913: 383) described *Centaurium spicatum* raça *elegans* based on plants from Beira Litoral. He differentiated it from the type subspecies by its slender stems with thin and quite divergent branches, and by its pedicellate and distant flowers, with calyx lobes very similar to each other. Among the materials of Sampaio kept in the Herbarium of Oporto University (PO), the only material identified as *Centaurium spicatum* raça *elegans* is the voucher numbered as "6749 G. S.". It contains four plants measuring 12–17 cm long, in an early flowering state, with 6–7 nodes in the stem, middle leaves 7–12 × 2.5–4.8 mm, cymes monochasial or dichasial with 3–4 nodes, and flowers 9–11 mm, with pedicels 1.2–4 mm, calyx 5.5–7 mm, and corolla with tube 6–7.5 mm and lobes 2.5–3 mm.

Two labels made out of strips of newspaper are included in this voucher (Fig. 9). In the upper label Sampaio wrote "*Ílhavo*: Ria. 30, 6°. 1901 // Existe tamben em Esmoriz. Tem um porte bem diverso [= very different] da E. spicata. / E' pequena, subglauca". In the lower label Sampaio wrote "E. spicata / precox, nob. Differe do typo por florescer mais cedo [= early], pelos / ramosi (?) quasi capillares, pelas flores mais distantes, pelos ra- / mos mais aberto-divergentes, pelos" In the lower corner of the right side Sampaio wrote "E. spicata / raç. elegans Samp. // Ílhavo: Ria", using *Erythraea*, a synonym of the more recent name *Centaurium*. The standard label of the Instituto de Botánica "Dr. Gonçalo Sampaio" of the University of Porto identifies the specimen, numbered as "6749 G. S.", as "Centaurium spicatum Frits. rac. elegans Samp.", collected on "30–VI–1901" in "Ílhavo: Ria" by "Gonçalo Sampaio". The measurements exhibited by the four plants in the voucher and the Sampaio's handwritten notes (Fig. 9) on the sheet are in agreement with the description given by Sampaio (1913: 383) for this taxon. On page 383 of the *Manual da Flora Portugueza*, Sampaio indicated a wide area, "Prados maritimos, de Esmoriz a Mira", where "Ílhavo" is located. In consequence, this voucher contains plants that are type specimens. The first plant on the right, measuring 15 cm high, with a whole stem and at least one mature flower, is chosen as the better plant for representing *Centaurium spicatum* raça *elegans* Samp. (Fig. 9).

Chromosome number: $2n = 42$ (here published for the first time).

Phenology: Flowering from end-May to late July; mature seeds are available from end-June to August.

Habitat: Temporarily flooded depressions and desiccated streams, coastal or inland lagoons, in brackish or sandy substrates; 0–700 m.

Distribution area: West Iberian Peninsula. Portugal: Beira litoral; Spain: Huelva, Valladolid and Sevilla provinces.

Addition specimens studied: PORTUGAL. Beira Litoral: De Esmoriz a Mira, 30 Jun 1901, *G. Sampaio* (PO-Samp). SPAIN. Huelva: Puebla de Guzmán, represa cercana al pueblo, 20 Jul 1989, *S. Silvestre* (SEV-214292). Villanueva de las Cruces, La Tiesa, 6 Jun 2002, *C. Santa-Bárbara and Z. Díaz Lifante* (SEV-214423). El Almendro, 2 Jul 2010, *V. Girón and Z. Díaz Lifante* (SEV-249968); ídem, *Z. Díaz Lifante and F. García Díaz*, 2 Aug 2015 (SEV-270212); ídem, Entre El Almendro y Alosno, proximidades a un curso de agua, 24 Jun 2016, *Z. Díaz Lifante and C. García Llama*s (SEV 270231). Entre Puebla de Guzmán y El Almendro, 7 Jun 2015, *Z. Díaz Lifante* (SEV-270205). Sevilla: Coria del Río, "Dehesa de la Atalaya", 5 Jun 2016, *C. Andrés Camacho, Z. Díaz Lifante, J. Díaz Fernández* and C. *García Llamas* (SEV 270230). Puebla del Río, "Dehesa de Abajo", Z. *Díaz Lifante, J. Díaz Fernández* and C. *García Llamas* (SEV 270229). Entre Sevilla y Montequinto, Campus de la Universidad Pablo de Olavide, 1 Jun 2015, *Z. Díaz Lifante* (SEV-270203). Valladolid: Aldeamayor de San Martín, 26 Jul 1983, *M. Ladero, F. Navarro and C. Valle* (SALAF-4797).

Conservation status: Not evaluated yet. In previous analyses, Díaz Lifante et al. (2018) indicated a variation in the number of plants per square meter between 0.51 (population of Atalaya, here named as ATA) and 25.9 (population of El Almendro, here named as ALM), with a significant amount of 6.79 in a human disturbed habit at the University Campus of Pablo Olavide. The tests of seed germination here carried out have shown that it is achieved easily in autumn at room temperature, so it seems that there is not a deep or complete dormancy mechanism. But it is urgent to develop a searching program in similar habitats in a more extensive area to complete the knowledge of its distribution, and to monitor populations evaluating its present threat *status* which would allow for a more accurate categorization of extinction risk (IUCN 2019).

Ecological observations: Plants of *V. elegans* blooms in early summer, occupying the margins of temporal stream beds, ponds and damp places when dried up and the competition with other herbaceous plants is low. The populations in Huelva and Valladolid provinces colonize soils formed on acid rocks, such as slates and granite, but the populations from Seville province grow in loamy soil.

In the location of Aldeamayor de San Martín, Villanueva de Las Cruces, and Puebla de Guzmán, *V. elegans* cohabits with *S. spicata*, but to date only in two locations, El Almendro (Huelva province, Spain) and Dehesa de Abajo (Sevilla province, Spain) *E. pusillum* is also found (Fig. 8a, b). It is very probable that the three taxa coexist in Beira Litoral province (Portugal), from where herbarium specimens have been studied. In the recent localization in wastelands in the Campus of the University Pablo de Olavide (Seville province), *V. elegans* occupies depressions retaining moisture for some time, very close to the alluvial terrace of the Guadaira river, near where this flows into the Guadalquivir river. There *V. elegans* cohabits with *S. spicata*, but this species flowers one month later.

Information on Electronic Supplementary Material

Online Resource 1. Specimens and accession number in GenBank for DNA sequences used in the molecular analyses.
Online Resource 2. Results for the PCA analysis: Eigenvalue, percentage and cumulative variance for the first 5 principal components, and eigenvectors for the continuous characters in them.
Online Resource 3. Results for the OSA analysis: Eigenvalue, percentage of variance for the two dimensions, and their eigenvectors for the nine categorical characters.
Online Resource 4. Results for the ANOVA analysis of the differences in 25 continuous characters among *Exaculum pusillum*, *Schenkia elegans* and *S. spicata*.
Online Resource 5. Mean and 95 % confidence interval for some of the characters showing significant differences at $p<0.0001$ in the ANOVA carried out to the population means of *Exaculum pusillum*, *Schenkia elegans* and *S. spicata*.
Online Resource 6. Maximum and minimum values of the chromosome length (in μm) for the largest and the lowest chromosome pairs in the karyogram, and values of the ratio (r) between both, following Stebbins (1971), per each population studied in *Exaculum pusillum*, *Schenkia spicata* and *S. elegans*. Range, mean and standard deviation (s.d.) of values are included for each taxon.
Online Resource 7. Phylogenetic tree of tribe Chironieae, inferred using ITS sequences.
Online Resource 8. Phylogenetic tree of tribe Chironieae, inferred using plastid sequences.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00606-023-01864-0.

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Author contribution ZDL framed the starting hypothesis and designed the experimental phase, taking part in the sampling and different analyses of every phase of the research, made the taxonomic revision, and wrote the first and the final draft integrating the contributions of the rest of authors. ME designed, developed and analysed the molecular analyses, contributing significantly to the taxonomic decision, so as to the writing and revision of the final draft. CAC put forward in the sampling and experiments in the field, in the seed germination experiments and contributed to the revision of the final draft. CG-L took part in the sampling and experiments in the field, the seed germination experiments, and in the morphologic and molecular analyses of the plants. JL and SC developed and edited the cytometry analyses and contributed significantly to writing and revision of the final draft. All of the authors read and approved the final manuscript of this article.

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Data availability statement All data generated or analysed during this study are included in this published article (and its supplementary information files). The DNA sequences generated during this research will be deposited in the Genbank.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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